Kavault™
(Type A medicated article/ avilamycin premix)

Environmental Assessment for the Use of Kavault™ to Prevent/Reduce Diarrhea in Nursery Pigs

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Table of Contents

List of Appendices

Kavault™ (avilamycin)

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1.0 Introduction

Kavault™ is a Type A medicated article-containing feed premix. Avilamycin is the active ingredient in Kavault™. The following assessment is provided to support an application for the use of avilamycin at a targeted dose of 80 ppm in the feed of nursery pigs for the prevention and/or reduction in incidence and severity of diarrhea caused by *Escherichia coli*.

This environmental risk assessment has been conducted based on the VICH guidelines for both phase I (VICH GL6) and phase II (VICH GL38).

2.0 Pattern of Use and Relevant Exposure Routes

Kavault™ will be administered to swine via feed at a maximum concentration of 80 ppm (80 mg avilamycin activity/kg feed) for a cycle of 21 days with the possibility of a second cycle of 21 days immediately following the first. Treatment will begin immediately after pigs are transferred into the nursery barn. Administration will be by veterinary prescription only. Up to one refill may be issued for a total possible duration of 42 days.

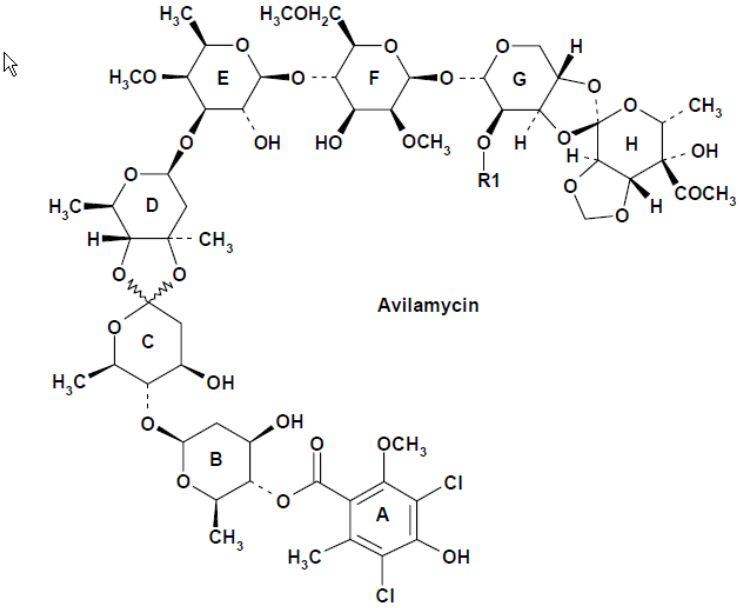
The primary route of environmental exposure to avilamycin will be from swine manure removed from storage as a liquid slurry and applied to agricultural land as fertilizer. While unlikely, any possible spillage and breakage of containers of Kavault™ or medicated feed might also be applied with manure to agricultural land. However, any additional environmental exposure is expected to be insignificant compared to that following from administration to pigs.

Runoff from swine facilities as a route of environmental exposure to avilamycin will not be considered because swine facilities are designed such that contaminated runoff is not expected. Manure storage is either covered such that rain does not enter or, in the case of uncovered storage facilities, the storage capacity can accommodate waste as well as rainfall events. State government regulations, including those for states which are primary swine producers, dictate the need to contain manure (e.g. Iowa IAC [567] 65.2; North Carolina 15A NCAC 02T.1307).

3.0 Description of the Product

The active ingredient in Kavault™ is avilamycin. Avilamycin is an antibiotic of the orthosomycin family consisting of a six-member oligosaccharide with dichloroiosoeverninic acid at one end and methyl eurekanate at the other. Avilamycin is produced by fermentation of *Streptomyces viridochromogenes* and is composed of a mixture of avilamycin A (>60%), avilamycin B (<18%) and 14 minor factors, with none of the minor avilamycins contributing more than 6% of the total avilamycin content.

Avilamycin is primarily active against gram positive bacteria through inhibition of protein synthesis by binding to the 50S ribosomal subunit and preventing the correct positioning of tRNA and initiation factor 2 (Kofoed and Vester, 2002).

International Non-proprietary Name (INN):	Avilamycin
Chemical Name:	O-(1R)-4-C-acetyl-6-deoxy-2,3-O-methylene-D-galactopyranosylidene-(1-3-4)-2-O-(2-methyl-1-oxopropyl)-α-L-lyxopyranosyl O-2,6-dideoxy-4-O-(3,5-dichloro-4-hydroxy-2-methoxy-6-methylbenzoyl)-β-D-arabino-hexopyranosyl-(1-4)-O-2,6-dideoxy-D-arabino-hexopyranosylidene-(1-3-4)-O-2,6-dideoxy-3-C-methyl-β-D-arabino-hexopyranosyl-(1-3)-O-6-deoxy-4-O-methyl-β-D-galactopyranosyl-(1-4)-2,6-di-O-methyl-β-D-mannopyranoside
CAS Number:	11051-71-1 (Avilamycin) 69787-79-7 (Avilamycin A)
Molecular Formula:	$C_{61}H_{88}Cl_2O_{32}$ (Avilamycin A)
Molecular Weight:	1404.2 (Avilamycin A)
Structural Formula:	

Avilamycin

Factor	R1	Other Modifications
A	$-COCH(CH_3)_2$	
A'	$-COCH_2CH_3$	–H replaces $-COCH_3$ on Ring H
B	$-COCH_3$	
E	–H	$-CHOHCH_3$ replaces $-COCH_3$ on Ring H
F	$-COCH(CH_3)_2$	–H replaces –Cl adjacent to $-OCH_3$ and –OH replaces $-OCH_3$ on Ring A
G	$-COC_4H_9$	
H	$-COCH(CH_3)_2$	–H replaces –Cl adjacent to $-OCH_3$ on Ring A
I	$-COCH_2CH_3$	
J	$-COCH(CH_3)_2$	–OH replaces $-OCH_3$ on Ring F

4.0 Phase I Environmental Impact Assessment

Final Guidance for Industry #89 (CVM, 2001) published by the FDA, Center for Veterinary Medicine, and the VICH GL6 Phase I guidance for Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) were consulted to conduct the Phase I Environmental Impact Assessment for the use of Kavault™ in swine. In this Phase I assessment, the maximum concentration of avilamycin in the manure and the soil has been calculated. No metabolism or degradation in manure is assumed and a total residue approach is taken for the Phase I assessment. The initiation of a Phase II assessment is dependent upon the trigger established in the VICH GL6 guidance: if the predicted environmental concentration of the total residue in soil is greater than 100 µg/kg, a Phase II assessment is warranted.

4.1 Calculation of Predicted Environmental Concentration

4.1.1 Calculation of concentration in manure

To estimate the concentration of avilamycin in swine manure, it was assumed that all pigs in the nursery barn were fed avilamycin continuously for 42 days (equivalent to a maximum of two dosing cycles). Nursery pigs enter and leave the nursery barn at average ages 19.4 and 64.8 days, respectively (USDA, 2008). Since the maximum treatment duration of 42 days is almost the average length of stay for a pig in the nursery barn (45.5 days), it will be assumed that pigs are fed for the duration of their stay in the nursery barn. The other assumptions used to calculate a manure concentration are in Table 1.

Table 1. Assumptions used to calculate avilamycin concentration in swine manure

Body Weight:	5 to 30 kg[a]
Concentration in Feed:	80 mg/kg
Daily Feed Intake:	0.067 $kg_{feed}/kg_{bodyweight}$[b]
Daily Manure Production:	0.08 $kg_{manure}/kg_{bodyweight}$[c]

[a]Based on pigs that are 3 to 10 weeks old. Age is based on USDA (2008). Size is from Schinckel et al (1997)

[b]Feed intake is calculated from NRC Nutrient Requirements of Swine (1998, Table 10-1) for pigs 5 to 20 kg based on average feed (750 g) intake divided by average body weight (12.5 kg)..

[c]The manure production value used here is consistent with reported values in ASAE (2003, Table 1); MWPS (2004, Table 6); and Ohio Manure Management Guide (2006, Table 1).

Thus, the concentration of total avilamycin residues (e.g. avilamycin plus any metabolites) in manure was calculated as:

$$[Avilamycin]_{manure} = \frac{Concentration\ in\ Feed \times Daily\ Feed\ Intake}{Daily\ Manure\ Production}$$

$$[Avilamycin]_{manure} = \frac{\frac{80\ mg}{kg_{feed}}\ X\ 0.067\ \frac{kg_{feed}}{kg_{bodyweight}}}{0.08\ \frac{kg_{manure}}{kg_{bodyweight}}} = 67\ mg/kg$$

4.1.2 Calculation of concentration in soil

The maximum concentration of avilamycin in the soil has been calculated using commonly used agronomic and manure management practices for application of swine manure to agricultural land.

Manure from swine in intensive pork production systems is collected, stored and land applied as a liquid slurry consisting of feces, urine, washwater and spilled drinking water. Manure from swine is applied to soil using injection or incorporation at an upper rate of 22,700 kg/acre. Manure is incorporated into soil to reduce nutrient loss, control odor, and reduce runoff even when a conservation tillage (e.g. "no-till") strategy is being used in a field. An incorporation depth of 15 cm is appropriate for both conventional and conservation tillage methods. Assuming an incorporation depth of 15 cm and an average bulk density of soil of 1500 kg/m^3, the weight of the soil in an acre into which the manure is mixed is approximately 910,500 kg:

$$Weight\ of\ Soil = 1\ acre\ \times 4047\frac{m^2}{acre} \times 0.15\ m \times 1500\ kg/m^3$$
$$= 910{,}500\ kg$$

Table 2. Assumptions used to calculate avilamycin concentration in soil

Application Rate of Swine Manure to Soil	22,700 kg/acre*
Plow Depth	15 cm*
Average Bulk Density of Soil	1500 kg/m^3

*These are traditionally used values in environmental assessments and reflect typical agricultural practices.

$$[Avilamycin]_{Soil} = \frac{Concentration\ in\ Manure\ \times Application\ Rate\ of\ Manure\ to\ Soil}{Weight\ of\ Soil/acre}$$

$$= \frac{67\ mg/kg \times 22700 kg/acre}{910{,}500 kg/acre} = 1.67\frac{mg}{kg} = 1{,}670\ \mu g/kg$$

Assuming no degradation in manure or soil, the concentration of avilamycin in soil after application of swine manure could be as high as 1,670 µg/kg. Since the initial concentration in the soil is more than 100 µg/kg, a Phase II environmental risk assessment was conducted, as per the VICH GL6 Final Guidance.

5.0 Phase II Environmental Impact Assessment

Final Guidance for Industry #166 (CVM, 2006) published by the FDA, Center for Veterinary Medicine, and the VICH GL38 Phase II guidance for Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) were consulted to conduct the Phase II Environmental Impact Assessment for the use of Kavault™ in swine. In the Phase II assessment, data with regard to the physical/chemical properties, environmental fate and environmental effects of avilamycin are used to assess the environmental risk of the use of avilamycin. Phase II progresses as two tiers; in the first (Tier A), a basic set of less complex studies is evaluated and is used to prepare a conservative risk assessment. If that risk assessment cannot rule out the possibility of a risk to the environment, more complex studies are then conducted and evaluated in Tier B. In the current assessment of the risk of use of avilamycin in swine, only a Tier A assessment has been conducted.

5.1 Summary of Available Data

This section reviews environmental data that has been collected with avilamycin. The Sponsor has collected data with avilamycin since the early 1980's.

5.1.1 Physical and Chemical Properties

The physical and chemical properties of avilamycin are listed in Table 3.

The aqueous solubility of avilamycin is pH-dependent with higher solubility at higher pH values. The solubility values for avilamycin A were determined to be < 0.125, 41 and 113 mg/L at pH 5, 7, and 9, respectively (Study I-EWD-82-07, 1982, Appendix A). The solubility values were also determined for a mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', and 0.9% D1+D2). The aqueous solubility of the avilamycin mixture was slightly higher than avilamycin A alone: < 0.125, 75, and 222 mg/L at pH 5, 7, and 9, respectively.

The logarithm of the octanol-water partition coefficient at pH 7.0 was determined to be 2.09 for avilamycin A and 1.94 for a mixture of avilamycin containing 28.7% avilamycin B (Study I-EWD-82-16, 1982, Appendix B). Avilamycin A appears to be slightly more lipophilic than the other avilamycin factors. In Study 66306 (2011, Appendix C), the octanol-water partition coefficient of avilamycin A was found to be related to pH and inversely proportional to its aqueous solubility. The log Pow of avilamycin A was determined to be 3.97, 2.55 and 0.681 at pH 4.5, 7, and 9, respectively.

The solubility and octanol-water partition data are consistent for a compound with an acidic dissociation constant. Additionally, the available data are in agreement that avilamycin A is more nonpolar than the other factors.

Table 3. Physical and Chemical Properties of Avilamycin

Melting Point (Merck Index, 1996)		Avilamycin A	Mixture of Avilamycin Factors
		181-182°C	188-189.5
Aqueous Solubility (I-EWD-82-07, 1982, Appendix A)		Avilamycin A	Mixture of Avilamycin Factors
	pH 5	< 0.125 mg/L	< 0.125 mg/L
	pH 7	41 mg/L	75 mg/L
	pH 9	113 mg/L	222 mg/L
n-Octanol/Water Partition Coefficient (Study 66306, 2011, Appendix C)	pH 4.5	pH 7	pH 9
	3.97	2.55	0.681
Dissociation Constant, pKa (internal data measured by potentiometric titration)	5.71, acidic		

5.1.2 Fate

The fate of avilamycin in pigs and in the environment is described in detail below. The environmental fate data collected with avilamycin is summarized in Table 4.

5.1.2.1 Metabolism and Excretion

Following dietary administration of radiolabeled avilamycin, over 95% of the dosed radioactivity is excreted in the feces and urine (Study ABC-0229, 1984 Appendix D). Of the excreted radioactivity, over 90% is recovered in feces while less than 10% is in urine (Study ABC-0229, 1984 Appendix D and Study ABC-0360, 1987, Appendix E). Avilamycin (mostly avilamycins A and B) makes up less than 5% of the total radioactive residue in the urine and feces (Study ABC-0309, 1985, Appendix F). Flambic acid, the major excreted metabolite is a result of the ortho ester cleavage linking of the C and D rings of avilamycin. Flambic acid interconverts with flambalactone and both have been identified when characterizing residues (Study ABC-0309, 1985, Appendix F; Study ABC-0371, 1987, Appendix G). Multiple swine and rat metabolism studies are summarized by Magnussen et al. (1991). The metabolism and excretion profile for rat is similar to that for swine.

5.1.2.1.1 Biological Activity of Avilamycin Metabolites

The metabolites of avilamycin have less pharmacological activity than avilamycin. In Studies I-EWD-81-13 and I-EWD-81-15 (1984, Appendix H), the feces were analyzed using two different techniques: a

gas chromatographic method that measured residue containing dichloroisoeverninic acid and a microbiological assay. The microbiological assay found at least five times less residues (in avilamycin equivalents) compared to the gas chromatography method. In Study ABC-0287 (1984, Appendix I), the liver from swine fed radiolabeled avilamycin was assayed for total radioactivity and bioautography. The mean bioactivity (expressed as avilamycin equivalents) was more than four times less than the total radioactive residues in liver expressed as avilamycin equivalents. Finally, the microbiological activity of purified flambic acid has been evaluated in gram positive bacteria (several strains each of *Costridium perfringens*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Staphylococcus aureus*, Study MR11MS-ELA, 2011, Appendix J). The minimum inhibitory concentration for avilamycin ranged from 0.25 to 8 µg/mL while no inhibitory activity was observed for flambic acid (tested as sodium flambate) at concentrations up to 128 µg/mL. Correcting for the difference in molecular weight, the activity of flambic acid is at least 40 times less than that of avilamycin.

5.1.2.2 Degradation

5.1.2.2.1 Hydrolysis

Avilamycin is not stable in water and undergoes hydrolysis more rapidly in acidic and basic media as compared to neutral conditions. The half-lives of avilamycin incubated in the dark at pH 5, 7, and 9 are 12, 230, and 52 hours, respectively (Study S-AAC-82-04, 1983, Appendix K). In this study, concentration was measured by a microbiological assay (with *Micrococcus flavus)*, therefore, the hydrolysis products were not microbiologically active.

5.1.2.2.2 Photolysis

Avilamycin is rapidly photodegraded in aqueous solutions. In Study S-AAC-82-04 (1983, Appendix L), solutions of avilamycin in pH 7 buffer were irradiated under fluorescent lamps which replicate an ultraviolet spectral energy distribution similar to natural sunlight. The first order degradation rate was calculated to be -0.59 hr^{-1} and the half life was 1.2 hours.

In Study EWD8429 (1984, Appendix M), the degradation rate of avilamycin was calculated to be 7.24 $days^{-1}$ in natural sunlight at 40°N latitude in the summer (partly cloudy on the day that this data was gathered). Using the light intensity in the study and the light intensity at various northerly latitudes, the half-lives under clear skies was calculated. The half-lives ranged from 0.086 days at 20°N in summer to 1.33 days at 50°N in winter.

In both photolysis studies above, the concentration of avilamycin was measured by a microbiological assay (with *Micrococcus flavus)*, therefore, the photolysis products of avilamycin are not microbiologically active. Avilamycin was rapidly degraded under experimental conditions in which the aqueous media was clear and shallow allowing maximal light penetration. In the surface waters impacted by agricultural runoff, degradation of avilamycin will likely be slower due to the presence of particles and dissolved materials as well as depth which limit light penetration.

Based on its hydrolytic and photolytic potential, avilamycin is not expected to persist in aqueous environments. However, because degradation rates are likely slower in real-world aqueous compartments, the environmental concentrations in the surface water will not be refined for photolysis and hydrolysis.

5.1.2.2.3 Degradation in Soil

The field dissipation of avilamycin residues was evaluated in Study ABC-0235 (1984, Appendix N). In this study, manure from pigs fed 90 ppm ^{14}C-avilamycin in feed was applied to the top 5 cm of a silty loam soil. Core samples up to 30 cm deep were evaluated for total radioactivity over the course of a year. The majority of the recovered radioactivity (>75%) was always found in the top 7.5 cm. After 4 weeks less than 50% of the applied radioactivity was found in the core samples.

A more recent degradation study of avilamycin in soils was conducted to fully evaluate the kinetics and identify major degradation products in four different soils (Study 66679, 2012, Appendix O). In this study, the soils were amended with ^{14}C-avilamycin at a rate of 1 mg/kg and incubated at 20°C under aerobic conditions in the dark. Evolved $^{14}CO_2$ was trapped using KOH and soils were extracted and extracts profiled using HPLC with radiometric detection. Major degradation products were identified using LC/MS/MS. Avilamycin was rapidly transformed in the soils: the maximum DT50 and DT90 values were 1.5 and 28.6 days, respectively. Additionally, there was significant mineralization; after 120 days, 15.1 to 42.7% of the applied radioactivity (AR) evolved as $^{14}CO_2$ in three of the soils, and in the fourth soil, 58.0% of the AR was evolved as $^{14}CO_2$ after just 51 days. A large amount of nonextractable residue was observed in the study. Since the three soils with the most $^{14}CO_2$ evolution had the highest nonextractable residue, it is likely that the residue is composed of small degradation products that were incorporated into the microbial-soil matrix. Six transformation products were observed that were greater than 10% of the applied radioactivity. The predominant degradation pathway was hydrolysis leading to the same cleavage of the orthoester linkage between the C and D rings in avilamycin similar to what is observed in metabolism studies. In the soil metabolism study, both flambic acid and

flambalactone were observed as well as the corresponding remaining moiety from the other side of the molecule. All three of these products experienced further degradation over the course of the study. Another minor pathway of degradation resulted in hydrolysis of the avilamycin at a different location and loss of the methyl eurekanate entity. This degradation product was also observed to further degrade. The only degradation product that did not clearly show further degradation during the study was found at levels greater than 10% in only one of the soils and only at the last three timepoints of the study, Days 73, 98 and 120. The structure of this degradation product was not determined, but the molecular weight was only 230 g/mole (versus 1404 g/mole for avilamycin A), therefore, this degradation product is unlikely to have the same bioactivity as avilamycin.

5.1.2.2.4 Degradation in Excreta

No studies have been conducted to evaluate the degradation of avilamycin in swine excreta or in manure slurry.

5.1.2.3 Soil Adsorption

The adsorption of avilamycin to soil and its potential soil mobility have been evaluated.

In Study EWD8609 (1986, Appendix P), a batch sorption study was conducted in which ^{14}C-avilamycin in 0.01 M $CaCl_2$ was equilibrated with three different soils (sandy loam, loam, and clay loam) for 16 hours. To reduce degradation during the study, the 0.01 M $CaCl_2$ was boiled to drive off CO2 to raise the pH. Also, the adsorption of avilamycin to glass was corrected by subtracting the amount that bound to glass in a soil-less control. The amount of radioactivity bound to soil was determined by subtraction of the supernatant from the amount added to the equilibration mixtures. The resulting Kd values were 51, 23, and 109 for the sandy loam, loam and clay loam soils, respectively.

In a more recent study, Study 66678 (2012, Appendix Q) following OECD guideline 106, ^{14}C-avilamycin was equilibrated with five different soils for up to 20 hours. The 0.01 M $CaCl_2$ was boiled to drive off dissolved CO_2 and the pH of the solution was adjusted to 7 to minimize degradation and increase solubility of avilamycin. Additionally, the test tubes were pre-conditioned with excess non-radiolabeled avilamycin to reduce adsorption to glass. The soil and aqueous phases were separated by centrifugation and Kd values for total radioactivity were calculated from the total radioactivity in the supernatant and the calculated amount in the soil by subtracting the supernatant radioactivity from what was originally added. For the five soils, the mean Kd values for total radioactivity ranged from 2.05 to 143 with an average of 54 and the average Koc value over the 5 soils was 3643. Additionally, the soil pellets were extracted and the extracts and the aqueous

supernatants were profiled by HPLC fractionation followed by liquid scintillation counting. Using the profile data, Kd values specific for avilamycin were calculated. For avilamycin the mean Kd values ranged from 2.01 to 29.3 with an average of 15 while the average Koc value was 1060. While the properties of avilamycin (instability especially at low pH values, low water solubility especially at low pH values, adsorption to glass) make it difficult to conduct a robust batch sorption study, the data from both of these studies can be taken as estimations of the binding of avilamycin to soil. Given the issues with the conduct of a batch sorption study with a compound possessing these characteristics, simulation of leaching may provide an alternative way to understand of the potential soil mobility of avilamycin.

In Study ABC-0337 (1986, Appendix R), the potential soil mobility of avilamycin and flambalactone were investigated using soil thin-layer chromatography. The distance that the compounds travelled in three different soils (coarse, medium, and fine) was compared to mobile and immobile reference standards. Avilamycin was classified as a low mobility compound. Flambalactone, which can be easily hydrolyzed to the more polar carboxylic acid (flambic acid), was found to be more mobile than avilamycin in the soils.

In the two studies in Study S-AAC-82-04 (1983, Appendix S), the mobility of avilamycin and its soil degradation products were investigated using soil columns. In the first study, four columns were prepared each with a different soil. ^{36}Cl-Avilamycin was applied to the top of the columns and then the columns were leached with water to simulate 60 cm of rainfall over approximately six days. Total amounts of radioactivity found in the leachates were 22.1, 53.3, 26.7, and 22.7% for the sand, sandy loam, loam and clay loam soils, respectively. In another part of this study, ^{36}Cl-avilamycin was aged for 30 days in the sandy loam soil, prior to placing that soil on the top of a soil column prepared with untreated sandy loam soil. Over 45 days, 40-mL of water per day, equivalent to 1.25 cm, was added to the tops of the columns and leachate collected. At the end of the study, 84.4% of the applied radioactivity was found in the leachate.

The studies together suggest that avilamycin can sorb to soil, but its rapidly-forming degradation products are likely to be more mobile in soil.

5.1.2.3.1 Bioconcentration

One way to estimate bioconcentration potential of a chemical is to consider its lipophilicity. The log Pow values for avilamycin range from 3.97 (at pH 4.5) to 0.681 (at pH 9). Veith and Kosian (1983) generated a linear model using a training set of 122 molecules to predict the bioconcentration factor for chemicals in fathead minnows:

$$\log BCF = (0.79 \times \log K_{ow}) - 0.40$$

Using this equation and the highest log Pow value for avilamycin (3.97), the estimated bioconcentration factor (BCF) for avilamycin is only 545. Bioaccumulation that affects the food chain typically becomes a concern with bioconcentration factors greater than 1000 or more. Additionally, the potential for avilamycin to bioaccumulate is very low given its limited half-life in water (due to hydrolysis and photolysis) and its propensity for metabolism across species evaluated (swine and rat).

Table 4. Environmental Fate of Avilamycin

<table>
<tr><td rowspan="5">Soil Adsorption/Desorption
(Study 66678, 2012, Appendix Q)</td><td></td><td colspan="2">Total Radioactivity Koc</td><td>Avilamycin Koc</td></tr>
<tr><td>Sand pH 5.2</td><td colspan="2">559</td><td>605</td></tr>
<tr><td>Loam pH 6.0</td><td colspan="2">3321</td><td>1325</td></tr>
<tr><td>Silty Clay Loam pH 6.3</td><td colspan="2">1987</td><td>537</td></tr>
<tr><td>Sandy Clay Loam pH 8.0</td><td colspan="2">397</td><td>388</td></tr>
<tr><td rowspan="3">Hydrolysis
(Study S-AAC-82-04, 1983, Appendix K)</td><td colspan="4">Hydrolytically unstable</td></tr>
<tr><td></td><td>pH 5</td><td>pH 7</td><td>pH 9</td></tr>
<tr><td>Half-life (hours)</td><td>12</td><td>230</td><td>52</td></tr>
<tr><td>Photolysis
(Study EWD8429, 1984, Appendix M)</td><td colspan="4">Half-lives range from 0.086 to 1.33 days from 20 to 50 degrees north latitude over summer and winter</td></tr>
<tr><td>Degradation in Soil for up to 120 days
(Study 66679, 2012, Appendix O)</td><td colspan="4">Biodegradation to $^{14}CO_2$ ranged from 15.1 to 58.0% of applied radioactivity. After 7 days less than 11% AR in all soils was identified as avilamycin. The DT50 and DT90 for avilamycin ranged from 0.2 to 1.5 days and 0.6 to 28.6 days, respectively. Six major degradation products were observed, including flambic acid and flambalactone which further degraded over the course of the study.</td></tr>
</table>

5.1.2.4 Toxicity

The environmental effects of avilamycin in the terrestrial and aquatic compartments are described below and summarized in Table 5.

5.1.2.4.1 Terrestrial Organisms

The toxicity of avilamycin to soil microflora, plants, and earthworms has been evaluated.

The effects of avilamycin on soil microflora, specifically carbon transformation (respiration) and nitrate formation (nitrification), were evaluated in Study 70541080 (2012, Appendix T). The initial soil concentrations of avilamycin (applied as crystalline avilamycin) were 1, 5, and 15 mg/kg soil (dry weight). There were no effects on respiration throughout the study. After 28 days, the carbon transformation rate in the highest treated group differed from the control rate by only 4%. There were transient effects on nitrification by avilamycin treatment. The data suggested that avilamycin treatment stimulated the formation of nitrate nitrogen early in the study. By Day 28, the nitrate formation rate in the controls had caught up to that in 1 and 5 mg avilamycin/kg treatments but not the 15 mg avilamycin/kg which was increased over the control by 34%. By Day 56 the difference in nitrate formation rate between the 15 mg avilamycin/kg and the control was only 11%. Therefore, no long-term impacts from avilamycin are expected on the soil microflora population.

An older study was conducted on the effects of avilamycin on the nitrification in soil (S-AAC-82-19, 1983, Appendix U). In the older study, the test substance was actually avilamycin residues applied as manure from pigs fed avilamycin at a concentration in the feed of 80 ppm. Manure was added to soil at rates that were equivalent to 13,658 and 27,315 kg/acre (as incorporated into the top 15 cm of soil, see Appendix U). The higher application rate is comparable to the typical application rate used to calculate the concentration of avilamycin residue in soil (Section 4.1.2, Table 2), so the concentration of avilamycin residue in the high rate was comparable to the calculated concentration in soil in this assessment. After 28 days, the amount of nitrate-nitrogen in the soil was the same as that in the feces-free soil control and only slightly higher than the soil control (≤18% difference) amended with the same amount of feces from pigs not treated with avilamycin.

Both the recent study (Study 70541080, 2012, Appendix T) and the older study (Study S-AAC-82-19, 1983, Appendix U) are in agreement that avilamycin and its residues are not expected to have a significant effect on soil microflora. This environmental risk assessment will be conducted using the data from the more recent study (Study 70541080, 2012) because it was conducted to current guidelines, utilizes crystalline avilamycin, and evaluates the effects of higher concentrations than those evaluated in the older study.

In a seedling germination test with six species, no seeds were affected by direct exposure to avilamycin via filter paper saturated with aqueous

avilamycin solutions (Study ABC-0263, 1984, Appendix V). The highest concentration of avilamycin in the aqueous solutions was calculated to expose seeds to a concentration of avilamycin activity equivalent to that resulting from the land application of 12,141 kg manure/acre when using manure from swine fed 80 ppm avilamycin. This application rate is half of the rate used in Section 4.1.2, Table 2.

The phytotoxicity of manure from pigs fed avilamycin (at 300 ppm in feed) was evaluated in Study ABC-0261 (1984, Appendix W). Seeds of corn, wheat, soybean, and tomato plants were grown in the amended soil. The application rate of manure to the soil was equivalent to 1642 kg fresh manure/acre (and based on avilamycin activity, was equivalent to 6158 kg/acre of manure from pigs fed 80 mg/kg avilamycin). The manure application rate of 6158 kg/acre is less than 30% of that used in typical agricultural practices (22,700 kg/acre, Section 4.1.2, Table 2). There were no detrimental effects on shoot height or weight. There was an increase (100% compared to the untreated soil) in the root weight in tomatoes and a decrease (27% compared to the untreated soil) in root weight of corn. Given the difficulty in removing the root mass completely and cleanly from the soil, it is possible that differences in this endpoint may be due to procedures used for the study. While an increase in root mass does not seem to be an adverse effect, the significance of a 27% decrease in root weight in the corn is unknown, especially when the above ground parameters were not affected.

The concentrations evaluated in the seed germination study and the older phytotoxicity study evaluated the toxicity of concentrations of avilamycin or avilamycin residues that were lower than those expected in the soil. Therefore, another phytotoxicity study has been conducted following the OECD 208 guideline to evaluate seed emergence and growth (Study 66369, 2012, Appendix X). In the new study, six crop species (corn, oat, radish, soybean, sugar beet, and tomato) were planted in avilamycin-fortified sandy loam soil. Avilamycin was added to soil as crystalline avilamycin at much higher concentrations than were tested in the previous studies and at higher concentrations than what is expected in soil. No effects were observed on percent emergence of the seeds, survival of seedlings after emergence, individual shoot length, and replicate shoot weight for any species up to the highest concentration tested, 500 mg avilamycin/kg soil (dry weight).

All three of the phytotoxicity studies are consistent with the conclusion that plants are not particularly sensitive to exposure to avilamycin. Since the concentrations tested in the older phytotoxicity study are not as high as the predicted environmental concentration in soil, this environmental risk assessment will be conducted using the data from the more recent study (Study 66369, 2012).

The effects of avilamycin on earthworms were evaluated in a subchronic growth and survival study and in a reproduction study. In Study W01882 (1988 Appendix Y), *Lumbricus terrestris* were exposed to avilamycin for 14 days with no adverse effects on survival, growth, or behavior observed at concentrations up to 100 mg avilamycin/kg in soil (dry weight). In a chronic study including a reproduction endpoint (Study 66370, 2011, Appendix Z), *Eisenia fetida* were exposed to avilamycin in soil up to a concentration of 1,300 mg avilamycin/kg soil (dry weight). After 4 weeks, adults were removed, leaving cocoons and any juveniles. After a second 4-week period, juvenile worms were counted to assess reproduction. No effects were observed on survival and growth of the adult worms. While there were no statistically significant differences in the number of juveniles found in the soil treated with avilamycin, there were 18 to 20% fewer earthworms in the two highest doses (670 and 1,300 mg/kg) compared with the pooled control. Based on these decreases, the NOEC was conservatively estimated to be 330 mg/kg. The results of the subchronic and reproduction studies are consistent. Therefore, this environmental risk assessment will be conducted using the data from the more recent study (Study 66370, 2011).

Given the rate of degradation of avilamycin in soil observed in Study 66679 (2012, Appendix O) in which DT50s ranged from 0.5 to 1.5 days for four soils, organisms in these terrestrial toxicity studies were likely exposed to significant concentrations of avilamycin degradation products, including flambic acid and/or flambalactone. No terrestrial organism tested appears to have a particular sensitivity to avilamycin or its residues.

5.1.2.4.2 Aquatic Organisms

The toxicity of avilamcyin to microorganisms, cyanobacteria, daphnia, and fish has been evaluated.

In Study S-AAC-82-08, 1983, Appendix AA), the toxicity of avilamycin to sewage microorganisms was investigated by repeatedly treating an activated sludge inoculum with increasing concentrations of avilamycin, up to 102.6 mg/L (theoretical), under aerobic conditions. The highest measured concentration of avilamycin activity in the test systems was 87.6 mg/L. Measurements of BOD, colony-forming units, pH, and solids content did not indicate that avilamycin would have a deleterious impact upon the digestive process of a diverse microbial population. The design of the study, increasing the theoretical concentration from 0.1 to 102.6 mg/L could have resulted in acclimation of the system to effects of avilamycin.

The inhibitory effects of avilamycin on *Synechococcus leopoliensis*, a photosynthetic cyanobacterium, have been evaluated in a 72 hour static

exposure (Study T4EFR0701, 2007, Appendix BB). The nominal concentrations were 0.625, 1.25, 2.5, 5.0 and 10 mg/L and the actual concentrations were not measured. Avilamycin caused a decrease in the growth rate which was more pronounced after 24 hours than at the end of the study (72 hours). This may reflect a decreasing concentration of bioactivity as avilamycin is degraded due to photolysis and/or hydrolysis. The EC50 values for growth rate and yield were determined to be >10 mg/L and 6.85 mg/L, respectively, based on nominal concentrations. The NOEC values for growth rate and yield were considered to be 2.5 and 1.25 mg/L, respectively. It is likely that avilamycin was degraded by hydrolysis and photolysis during this study in which the aqueous cultures were exposed to 72 hours of constant illumination of 3700 lux. In Studies S-AAC-82-04 (Appendix L) and EWD8429 (Appendix M), the half-life of avilamycin was determined to be as low as 1.2 hours in aqueous solutions exposed to simulated or actual sunlight. Hydrolysis occurs at a slower rate when solutions of avilamycin are incubated in the dark with half-lives of 230 and 52 hours at pH values of 7 and 9, respectively (in the cyanobacteria study the pH ranged from 8.07 to 8.25), in Study S-AAC-82-04 (Appendix K). If the faster hydrolysis rate is considered, approximately 60% of the avilamycin would hydrolyze over the 72 hour study. The amount that would photolyze is more difficult to estimate. Therefore, the average true exposure concentrations during the cyanobacteria study are likely much lower than the nominal concentrations upon which the EC50 and NOEC values are based. Adding to the uncertainty in the relationship of toxicity to exposure is that, in considering a realistic aqueous environment in which avilamycin residues run off into surface water, hydrolysis and photolysis will also occur, although photolysis will likely be attenuated due to lower light penetration.

To evaluate the toxicity of avilamycin to aquatic invertebrates, two acute toxicity studies with *Daphnia magna* have been conducted. In Study C03382 (1983, Appendix CC), daphnia were exposed to avilamycin (as dried fermentation product) in a static toxicity test for 48 hours at a single concentration. The nominal concentration was 100 mg/L but the measured concentrations ranged from 18.6 to 29.0 mg/L over the test with a mean measured concentration of 23.8 mg/L (concentrations were measured as antimicrobial activity). The disparity between the nominal and measured concentrations was likely due to a combination of the susceptibility of avilamycin to hydrolysis and photolysis (especially during the prolonged pre-study stirring period) and the limited solubility of the dried fermentation product. No mortality, immobilization or sublethal effects were observed. The EC50 was >23.8 mg/L and the NOEC was 23.8 mg/L. Recently, another acute toxicity study with daphnia exposed to avilamycin was conducted in order to confirm that the material was not toxic to daphnids at higher concentrations by using crystalline avilamycin (Study 66272, 2011, Appendix DD). Daphnids

were exposed to nominal concentrations up to 160 mg/L and again no toxicity was noted in any daphnid at any concentration. The mean measured concentrations were slightly lower than nominal and the highest treatment level was measured to be 138 mg/L (concentrations were measured using an HPLC/uv method). Therefore, the EC50 was >138 mg/L and the NOEC was 138 mg/L. In Study 66272, there was little evidence of degradation under the relatively low light conditions of 604 lux with a 16 h:8 h light:dark cycle. This environmental risk assessment will be conducted using the data from the more recent study (Study 66272, 2011) because higher concentrations were tested using crystalline avilamycin and because the nominal and measured concentrations were similar. Therefore, the results are more reliable.

In Studies F12782 (1983, Appendix EE) and F12682 (1983, Appendix FF), bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) were exposed to avilamycin (as dried fermentation product) in static toxicity tests for 96 hours at a mean measured concentration of 35.4 mg/L (bluegill) and up to 47.8 mg/L (rainbow trout). Concentrations were measured as antimicrobial activity. For both of these studies, the nominal maximum concentration tested was 100 mg/L. As with Study C03382 (Appendix CC), the disparity between the nominal and measured concentrations was likely due to a combination of instability of avilamycin in water and in light as well as low water solubility of the dried fermentation product. The pH values measured during the tests ranged from 7.9 to 8.7. Therefore, it is likely that the bluegill and rainbow trout would have been exposed to some of the degradation products of avilamycin. No mortality or signs of toxicity were noted in any fish of either species. Therefore, for both species the LC50 is greater than the highest concentration tested and the NOEC is equal to the highest concentration tested.

The photolysis and hydrolysis products of avilamycin have not been identified. However, it is likely that some of them are the same as the transformation products observed in the soil degradation study since hydrolysis was a component of the degradation pathway in soil. Therefore, it cannot be ruled out that the aquatic species were exposed to some degree to the degradation products of avilamycin.

Table 5. Environmental Effects of Avilamycin

Terrestrial Effects Studies			
Soil Microflora Respiration and Nitrogen Transformation Tests (56 days) (Study 70541080, 2012, Appendix T)	Concentration as high as 15,000 µg/kg resulted in less than 25% difference from control after 56 days		
Terrestrial Plants – Emergence and Seedling Growth (Study 66369, 2012, Appendix X)		µg/kg	
		EC50/LC50	NOEC
	Corn	>500,000	500,000
	Oat	>500,000	500,000
	Radish	>500,000	500,000
	Soybean	>500,000	500,000
	Sugar beet	>500,000	500,000
	Tomato	>500,000	500,000
Earthworm Reproduction (Study 66370, 2011, Appendix Z)	NOEC 330,000 µg/kg		
Aquatic Effects Studies			
Inhibition of Sewage Microorganisms (Study S-AAC-82-08, 1983, Appendix AA)	Concentrations of avilamycin up to 87,600 µg/L had no significant effect on sewage microorganisms.		
Blue-green Algae (Cyanobacterial) Growth Inhibition (Study T4EFR0701, 2007, Appendix BB)		µg/L	
		Yield	Growth Rate
	EC50	6,850	>10,000
	NOEC	1,250	2,500
Daphnia immobilization (Study 66272, 2011, Appendix DD	EC50: >138,000 µg/L NOEC: 138,000 µg/L		
Fish Acute Toxicity (Studies F12782 and F12682, 1983, Appendix EE and Appendix FF)	Bluegill LC50: >35,400 µg/L NOEC: 35,400 µg/L Rainbow Trout LC50: >47,800 µg/L NOEC: 47,800 µg/L		

5.2 PEC Calculations and Refinements (Exposure Assessment)

5.2.1 Soil

The initial PEC_{soil} was calculated in the Phase I assessment as 1670 µg/kg.

The PEC_{manure} concentration was first calculated using the following assumptions: all pigs in a nursery barn will be treated for their duration in the nursery barn (since that duration can be almost as short as two consecutive 21-day treatments) and all residue eliminated is avilamycin or metabolite as active as avilamycin. The PEC calculation also assumes that the liquid manure slurry from the nursery

barn is applied to soil without dilution by manure from non-treated pigs. The PEC_{manure} of total avilamycin residues using these assumptions was 67 mg/kg.

Per the VICH guideline (GL38), the PEC_{manure} could be refined based on the actual composition of the dose excreted by the treated animal by adding the active substance and the relevant metabolites (those that are 10% or more of the administered dose). The data from several metabolism studies indicate that 1) very little intact avilamycin (<10% of dosed) is excreted, 2) the residue that is excreted is not microbiologically active, and 3) flambic acid (and maybe flambalactone) are components of the residue; however, the identity and quantity of other metabolites are unknown. Therefore, while the maximum concentration of avilamycin itself in manure is less than 6.7 mg/kg, a total residue approach will be used in this assessment, assuming that the avilamycin residues are no more biologically active than the parent molecule. The PEC_{manure} and PEC_{soil} will be considered to be 67 mg/kg and 1670 µg/kg, respectively.

Avilamycin degrades rapidly in soil to several degradation products which in turn break down, eventually to CO_2, with observed half-lives ranging from 0.2 to 1.5 days. Therefore, there is no concern that avilamycin residues will persist and build up in the terrestrial environment.

5.2.2 Groundwater

Avilamycin and its residues are moderately sorbed to soil, with mean Koc values ranging from 399 to 11952 in various soils (Study 66678, 2012; Appendix Q). Substantial proportions of avilamycin are degraded to CO_2 in soil, with most of the remaining residues undergoing primary metabolism. It is very unlikely that significant levels of avilamycin or active residues would be found in groundwater.

5.2.3 Surface Water

Movement of avilamycin from soil to surface water may occur through runoff following rainfall events. A scenario of 1% runoff of compound from 10 acres of soil into a one-acre pond which is 2 m deep was considered. A one-acre pond that is 2 m deep has a volume of 8,100,000 L. Inserting the concentration of avilamycin residues in manure (67 mg/kg) and the application rate of swine manure per acre (22,700 kg/acre), the following calculation was performed to estimate the maximum concentration of avilamycin residues in the pond:

$$[Avilamycin\ residues]_{pond} = \frac{[Avilamycin\ residues]_{manure} \times Application\ Rate\ \times 10\ acres\ \times 0.01}{8{,}100{,}000\ L}$$

$$[Avilamycin\ residues]_{pond} = \frac{67\frac{mg}{kg} \times 22700\frac{kg}{acre}\ \times 10\ acres\ \times 0.01}{8{,}100{,}000\ L} = 18.8\ \mu g/L$$

The concentration of total residues of avilamycin in swine manure is 67 mg/kg. A total of 22,700 kg of swine manure is applied per acre, such that 15,209,000 mg of avilamycin residues will be applied per 10 acres. Therefore, 152,090 mg would enter the pond. A one-acre pond that is 2 m deep has a volume of 8,100,000 L. Therefore, the concentration of total avilamycin residues in the pond, $PEC_{surface\ water}$, would be 18.8 µg/L.

The measured Koc values for avilamycin residues to soil ranged from 399 to 11952. While, the $PEC_{surface\ water}$ might be somewhat lower due to binding to sediments suspended in the pond, for purposes of this risk assessment, no consideration for adsorption to soil is used to refine the $PEC_{surface\ water}$:

Table 6 summarized the maximum predicted environmental concentrations for avilamycin residues in the terrestrial and aquatic compartments.

Table 6. Summary of PEC Calculations for Total Residues of Avilamycin

Compartment	Scenario	Concentration
Terrestrial	$PEC_{soil,\ total\ residues}$	1670 µg/kg
Aquatic	$PEC_{surface\ water,\ total\ residues}$	18.8 µg/L

5.3 PNEC Calculations (Effect Assessment)

In accordance with VICH GL38 Phase II guidance for Environmental Impact Assessments (EIA's), predicted no-effect concentrations were calculated using the recommended data set and the appropriate assessment factors.

5.3.1 Terrestrial

The assessment factors used and the calculated PNECs for terrestrial species are included in Table 7. Terrestrial species were not especially sensitive to avilamycin, which may reflect the rapid degradation of avilamycin in soil. Additionally, results of the soil degradation study indicate that terrestrial species tested for toxicity were probably exposed not only to avilamycin but also to significant concentrations of the predominant metabolite in swine excreta, flambic acid. The lowest PNEC value was in plants: 5,000 µg/kg.

Table 7. Terrestrial PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Soil Microflora	≤25% change from control = 15,000 µg/kg	1	15,000 µg/kg
Plants, growth – soil	EC50 > 500,000 µg/kg	100	5,000 µg/kg
Earthworms	NOEC = 330,000 µg/kg	10	33,000 µg/kg

5.3.2 Aquatic

The assessment factors used and the PNECs calculated for aquatic species are included in Table 8. Cyanobacteria (i.e., blue green algae) appear to be more susceptible to toxicity from avilamycin than fish or daphnids based on the median effective concentration. Additionally, while the NOEC for cyanobacteria is reported as 1250 µg/L, this value is based on the initial nominal concentrations, which likely declined under the conditions of the study. Because the average exposure concentrations in the cyanobacteria study were likely lower than the initial nominal concentrations, an additional assessment factor has been used in the calculation of the PNEC value to account for this difference. An assessment factor of 3 will be used to account for the difference between nominal and actual concentrations in addition to the typical default assessment factor of 100. Therefore, a total assessment factor of 300 was applied to the EC50 for cyanobacteria. The lowest PNEC value for aquatic species is in cyanobacteria: 22.8 µg/L.

Table 8. Aquatic PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Blue-green Algae (Cyanobacterial) Growth	EC50 = 6,850 µg/L NOEC = 1,250 µg/L	300	22.8 µg/L
Daphnia acute	EC50 > 138,000 µg/L	1000	138 µg/L
Fish Acute (bluegill)	LC50 > 35,400 µg/L	1000	35.4 µg/L

5.4 Risk Characterization

5.4.1 Terrestrial Compartment

The predicted maximum concentration of total residues of avilamycin in soil (PEC_{soil}) after a single application is 1670 µg/kg. The lowest terrestrial PNEC value for avilamycin was 5,000 µg/kg.

The PEC/PNEC ratio for the terrestrial compartment is 0.33 (Table 9). The ratio is less than one indicating that there is no significant risk to plants or other soil-dwelling species. Given the rapid degradation of avilamycin in soil, there is no concern for significant risk following repeated applications of manure from pigs fed avilamycin.

Table 9. PEC/PNEC Ratio for Terrestrial Compartment

Species	PEC_{soil}	PNEC	PEC/PNEC Ratio
Plants	Total avilamycin residues: 1670 µg/kg	>5000 µg/kg	0.33

5.4.2 Aquatic Compartment

The maximum predicted concentration of avilamycin residues in surface water is 18.8 µg/L. The lowest aquatic PNEC value calculated for avilamycin was 22.8 µg/L.

The PEC/PNEC ratio for the aquatic compartment is 0.82 (Table 10). The ratio is less than one, indicating that there is no significant risk to blue-green algae (cyanobacteria) or other aquatic- dwelling species. Given the potential for photolysis and hydrolysis, there is no significant risk for accumulation of avilamycin in the aquatic compartment.

Table 10. PEC/PNEC Ratios for Surface water Compartment

Species	$PEC_{surface\ water}$	PNEC	PEC/PNEC Ratio
Blue-green algae (cyanobacteria)	Total avilamycin residues: 18.8 µg/L	22.8 µg/L	0.82

5.5 Summary and Conclusions

The environmental impact from the administration of avilamycin (as Kavault™) via feed at a targeted concentration of 80 mg/kg for up to 42 days to control diarrhea in nursery pigs has been evaluated. The evaluation included review of a base set of data collected on the physical/chemical properties, environmental fate, and environmental effects of avilamycin and its degradation products. The pathway for introduction of avilamycin into the environment was via the application of swine manure as fertilizer to soil. Runoff to surface water from soil fertilized with swine excreta containing avilamycin residues was also considered. The risk assessment utilized worst case assumptions of the maximum number of days of administration as well as a total residue approach.

The maximum predicted concentration of total avilamycin residues in the soil is 1670 µg/kg and the maximum predicted concentration in surface water following runoff is 18.8 µg/L. The lowest predicted no effect concentration in the terrestrial compartment is calculated to be 5,000 µg/kg in soil (based on plants) and the lowest predicted no effect concentration in surface water is 22.8 µg/L (based on blue-green algae). In both compartments the predicted environmental concentrations are lower than the predicted no effect concentration. The PEC/PNEC ratios are 0.33 and 0.82 for terrestrial and aquatic compartments, respectively. Since avilamycin is extensively metabolized by animals and extensively degraded in soil and water, it is not expected to persist in the environment or accumulate in environmental species.

The avilamycin PEC/PNEC ratios are less than one for the terrestrial and aquatic compartments, and avilamycin will not persist or accumulate in the environment. Therefore, the use of Kavault™ in nursery swine is not expected to result in environmental impact through the application of swine manure to cropland soil. The

PEC/PNEC ratios are sufficiently low such that even if there were some spillage of the product which was then disposed of into the manure containment system and applied to land with the manure, adverse effects would be unlikely.

Because this conservative risk assessment has concluded that there is no expected harm to the environment, further data collection (e.g. a Tier B assessment) is not required.

6.0 Information on Environmental Assessment Expert

The following individual is responsible for the information in the Environmental Assessment Report for avilamycin used as Kavault™ Type A Medicated Article to control diarrhea in swine:

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Principal Research Scientist, Environmental Risk Assessment, Lilly Research Laboratories (2010 to present)

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Senior Toxicologist, Senior Research Scientist, Environmental Science/Nonclinical Safety Assessment, Lilly Research Laboratories (2000 to 2005)

Editorial Board of Environmental Toxicology and Chemistry

Research Scientist, Research Institute of Pharmaceutical Sciences, U. Mississippi (1996 to 1999)

Publications:

Fifteen publications and numerous presentations and posters in the field of environmental toxicology.

7.0 References

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Appendices

Appendix A - Study I-EWD-82-07: The solubility of avilamycin in aqueous solutions. Report Date: 1982.

Performing Laboratory: Lilly Research Laboratories

Test Articles:

Crystalline Avilamycin A
Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Excess test article was added to pH 5.0, 7.0 and 9.0 buffers. The solutions were shaken at 25°C and aliquots were removed for analysis periodically. The samples were filtered through a 20 micron filter and the filtrate was assayed in triplicate using a micro-biological agar well plate method.

Results:
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	Maximum solubility mg/L (timepoint achieved)	
	Avilamycin A	Mixture of Factors
pH 5.0	<0.125 mg/L	<0.125 mg/L
pH 7.0	41 mg/L (3 hrs)	75 mg/L (24 hrs)
pH 9.0	113 mg/L (3 hrs)	222 mg/L (3 hrs)

At pH 5.0, the solubilities of avilamycin A and the mixture were both less than 0.125 mg/L which was the limit of detection for the assay. At pH values of 7.0 and 9.0, after reaching maximum solubility, the avilamycin concentrations decreased, apparently due to hydrolysis. The results suggest that avilamycin A is slightly less water soluble than the other avilamycin factors.

Appendix B- Study I-EWD-82-16: n-Octanol/Water Partition Coefficient of Avilamycin (EL-750). Report Date: 1982.

Performing Laboratory: Lilly Research Laboratories

Test Article:

Crystalline Avilamycin A
Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Solutions of avilamycin in n-octanol at two different concentrations were equilibrated with pH 7.0 buffer at a temperature of 25°C for one hour. The phases were separated by centrifugation for 20 minutes and the concentration of avilamycin in each phase was analyzed using a microbiological agar well plate assay.

Results:

	Mean Log Kow at pH 7.0
Avilamycin A	2.09
Mixture of Avilamycin Factors	1.94

The results indicate that avilamycin A is likely more nonpolar than avilamycin B.

Appendix C - Study 66306: Avilamycin: Determination of n-octanol/water partition coefficient (shake flask method). Report Date: 2011.

Performing Laboratory: ABC Laboratories, Inc.

Guidelines: GLP, OECD 107

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Solutions of avilamycin in n-octanol were equilibrated with aqueous buffers having pH levels of 4.5, 7, and 9 at a temperature of 20°C. The concentration of avilamycin A in each phase was determined by LC/MS/MS.

Results:

	Log Pow* (standard deviation)
pH 4.5	3.97 (0.12)
pH 7	2.55 (0.05)
pH 9	0.681 (0.023)

While the test article was a mixture of avilamycin factors, the LC/MS/MS method specifically measured avilamycin A. Therefore, the log Pow values reported are for avilamycin A.

*The partition coefficient values are not necessarily for the neutral species. Therefore, instead of Kow values which implies the partition coefficient of the neutral species, they have been reported as Pow values.

Appendix D - Study ABC-0229: ^{14}C Avilamycin balance-excretion study in swine. Report Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: ^{14}C Avilamycin fermented from uniformly labeled glucose

Methods:

Two crossbred gilts were housed in metabolism cages and fed twice daily with 0.9 kg of swine finisher diet containing unlabeled avilamycin at 60 ppm of microbiological activity for seven days. At the end of this predosing, each pig received a one-time dose of 120 mg ^{14}C avilamycin incorporated into 450 g of ration. Animals were then fed unmedicated ration for the duration of the experiment. Complete urine and fecal collections were made. Samples of urine were analyzed by liquid scintillation counting. Samples of feces were analyzed by combustion.

Results:

Gilt 594 excreted 96.9% of the radioactive dose over the collection period, while gilt 596 excreted 99.0%. The percentage of the total excreted radioactivity in feces and urine is tabulated below.

	Gilt 594	Gilt 596	Average
Feces	92.73%	94.06%	93.4%
Urine	4.15%	4.98%	4.6%

Appendix E - Study ABC-0360: A steady-state tissue residue study in swine dosed with uniformly labeled ^{14}C avilamycin. Report Date: 1987.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: ^{14}C Avilamycin fermented from uniformly labeled glucose

Methods:

Six crossbred swine, four barrows and two gilts, weighing approximately 44 kg each were fed at 12-hour intervals for either ten or fourteen days with a ration containing a nominal concentration of 60 mg ^{14}C avilamycin per kilogram of feed. Each day, animals received an amount of ration equal to 4% of their body weights. Radioactivity in the excreta of one barrow was assayed to estimate steady-state concentrations in feces and proportions in urine and feces.

Results:

There was approximately 92% of the recovered dose in the feces and 8% in the urine. Concentration of radioactivity (^{14}C avilamycin equivalents) in feces was approximately 120 ppm for the 60 mg/kg dosing level.

Appendix F - Study ABC-0309: Characterization of ^{14}C avilamycin residues in swine liver and excreta. Report Date: 1985.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: Liver and excreta from swine fed ^{14}C avilamycin

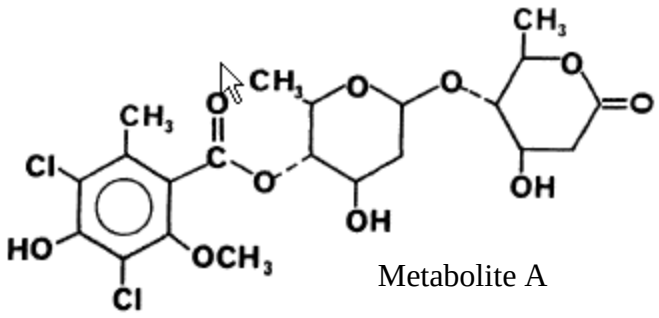
Methods:

^{14}C Avilamycin was synthesized by fermentation using ^{14}C diethylmalonate as precursor. Swine weighing approximately 44 kg were dosed at a level of 80 ppm ^{14}C avilamcyin in the feed for a period of at least seven days in a steady state tissue residue study (ABC-0287). Excreta samples were assayed to determine the concentrations of radioactivity and the nature of radioactive residues. Radioactivity was extracted from the feces with acetone/water. Both the feces extracts and the urine were partitioned into ethyl acetate which was done first under neutral conditions and then under acidic conditions. Various partition and chromatographic procedures were used for separation and purification. Specific metabolites were characterized by proton NMR and by negative fast atom bombardment (FAB) mass spectroscopy.

Results:

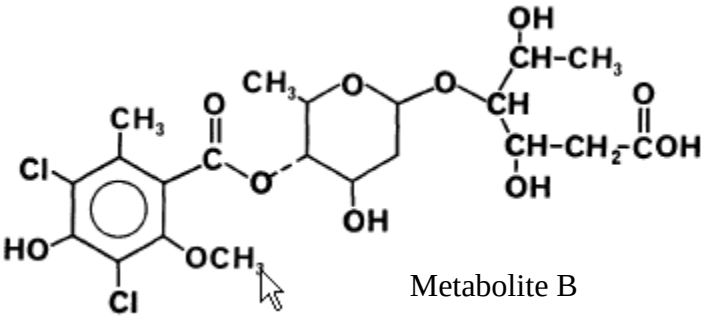
Almost 90% of the excreted radioactivity was extractable. Approximately 10% of the excreted activity partitioned into ethyl acetate under neutral pH, while 74 to 84% was extractable after adjustment to a pH range of 2 to 4.5. Less than 5% of the excreted radioactivity was identified as known avilamycin factors.

Metabolite A was determined to be formed as a result of cleavage of the orthoester linking the C and D rings of avilamycin resulting in a lactone structure shown in the figure below. Quantitation work showed that this metabolite represents 40 to 50% of the total radioactive residue in urine and feces.



Metabolite A

Another metabolite, Metabolite B, was acidic in nature and was observed to partially convert to Metabolite A. The structure of Metabolite B is in the figure below.



Metabolite B

In later studies, it was observed that Metabolite B is the actual metabolite, but that it can be converted to Metabolite A. Metabolite B is known as flambic acid and Metabolite A as flambalactone.

Appendix G - Study ABC-0371: Comparative metabolism of ^{14}C avilamycin in swine and rats. Study Date: 1987.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: ^{14}C Avilamycin fermented from uniformly labeled glucose

Methods:
Six rats, three males and three females, were fed a ration containing 550 ppm of uniformly labeled ^{14}C avilamycin for 4.5 days. Urine and feces were collected during the dosing period and livers were collected at sacrifice. Urine, feces, and liver tissue from swine fed the same lot of ^{14}C avilamycin were obtained from a previous experiment (ABC-0360). Fractionation and chromatographic comparisons were made between samples from treated rats and corresponding samples from treated swine.

Results:
The metabolite pattern in urine and feces of treated swine was essentially the same as the pattern for rats. The most abundant metabolite was flambic acid. There were three fecal metabolites which were derived from the oligosaccharide and eurekanate portion of avilamycin. Parent avilamycin constituted less than 10% of the fecal radioactivity in pigs.

The metabolite profiles in livers of treated rats and pigs were essentially the same. Flambic acid was the most abundant metabolite. Parent avilamycin concentrations in rat and pig livers were less than 0.05 mg/kg. The pattern of minor metabolites was similar, but none of the minor metabolites were sufficiently abundant for identification.

Appendix H - Study I-EWD-81-13 and Study I-EWD-81-15: Determination of residues in the feces of swine fed diets containing avilamycin. Study Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Test Article: Feces from starter pigs fed diets containing 20 ppm avilamycin

Methods:
Feces from starter pigs fed standard diets containing 20 ppm of avilamycin in three different product forms for a period of six days were assayed using microbiological and gas chromatographic residue methods. The microbiological assay measured activity against *Micrococcus flavus*. The gas chromatographic assay detected dichloroisoeverninic acid, so that it measured the total residue of avilamycin plus any degradation products that hydrolyze to dichloroisoeverninic acid. The microbiological assay was conducted on two replicates from each treatment while gas chromatography was done on a single replicate.

Results:
For the single replicate which was evaluated by both microbiological activity and gas chromatography, the microbiological results represent 2.0%, 4.5% and 15.0% of the total residue for the crystalline, micronized, and nonmicronized product forms, respectively.

Treatment Group	Pen Number	Assay Results		Activity as % of total residues
		GC (ppm as avilamycin equivalents)	Microbiological (ppm)	
Crystalline	5	43.3	0.88	2.0
	25	N/A	1.0	--
Micronized	1	40.1	1.80	4.5
	30	N/A	2.76	--
Nonmicronized	4	43.4	6.50	15.0
	31	N/A	10.40	--
Control	29	NDR	NDR	--
	34	N/A	NDR	--

N/A – information not available
NDR – no detectable residue (detection limit of 2 ppm for GC and 0.03 ppm for microbiological assay)

Appendix I - Study ABC-0287: ^{14}C Avilamycin steady-state tissue residue study in swine. Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: ^{14}C Avilamycin obtained by fermentation starting with ^{14}C-diethylmalonate as a precursor; more than 85% of the label is in the dichloroisoeverninic moiety

Methods:
Nine crossbred swine, five barrows and four gilts, weighing approximately 44 kg each were fed at 12-hour intervals for either four, seven, or ten days with a ration containing 76.19 mg ^{14}C avilamycin per kilogram of feed. Each day, animals received an amount of ration equal to 4% of their body weights. This resulted in a daily dose of 134 mg of ^{14}C avilamycin per animal. Al animals were sacrificed at a practical zero-time withdrawal of six hours after the final feeding. Muscle, liver, kidney, fat, and bile were collected for radiochemical analyses. Selected tissues were assayed for parent avilamycin by bioautography and for residues containing dichloroisoeverninic acid moiety. Radioactivity in one barrow was assayed to estimate steady-state concentrations in feces and proportions in urine and feces.

Results:
After ten days of dosing, total mean radioactivity residues in liver, fat, and kidney, expressed as avilamycin equivalents, were 0.22, 0.12, and 0.10 mg/kg, respectively. Residues in muscle were less than 0.025 mg/kg. Steady state concentrations of radioactivity were attained in muscle, liver, and kidney within four days after the initiation of dosing. A steady state concentration was not attained in fat during this study.

Liver, kidney, and fat from animals dosed for ten days were assayed for parent avilamycin by bioautography. No residues were detected in either kidney or fat, while only a trace of avilamycin (less than 0.05 mg/kg) was found in liver. These same tissues were also assayed for residues containing the dichloroisoeverninic moiety. Both liver and kidney contained detectable levels of dichloroisoeverninic-related residues, with the levels in liver representing 50% or more of the total radioactivity residue. No dichloroisoeverninic-related residues were detected in fat.

Therefore, while liver had 0.22 mg/kg avilamycin equivalents based on radioactivity; there was less than 0.05 mg/kg avilamycin equivalents based on bioactivity. This indicates that the metabolized avilamycin residue is more than four times less active than that of avilamycin.

Appendix J - Study MR11MS-ELA: Determination of the minimal inhibitory concentration of avilamycin and flambic acid against various bacteria. Study Date: 2011.

Performing Laboratory: Microbial Research, Inc.

Guidelines: Susceptibility testing conducted in the spirit of Standards M11-A7 and M31-A3 from the Clinical and Laboratory Standards Institute

Test Articles: Avilamycin and Sodium Flambate

Methods:
The bacteria tested included several strains of each of *Costridium perfringens*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Staphylococcus aureus*. Ninety-six well plates for MIC testing were prepared with either SBB (*Clostridium perfringens*) or MHB (Enterococci and Staphylococci) media. Avilamycin and flambic acid (as sodium flambate) were dissolved in methanol and added to the wells at various concentrations. The concentrations of each test item ranged from 0.06 to 128 µg/mL. The wells were inoculated with the appropriate bacteria and incubated for 46 to 48 hours under anaerobic (for *Clostridium perfringens*) conditions or 16 to 20 hours under aerobic (for Enterococci and Staphylococci) conditions. At the end of incubation, growth in the wells was assessed.

Results:

	Avilamycin	**Flambic Acid**
***Clostridium perfringens* (10)**		
MIC range	0.25 to 2 µg/mL	>128 µg/mL
***Enterococcus faecalis* (10)** ***Enterococcus faecium* (10)**		
MIC range	1 to 8 µg/mL	>128 µg/mL
***Staphylococcus aureus* (10)**		
MIC range	4 to 8 µg/mL	>128 µg/mL

No microbiological activity was observed with flambic acid at even the highest concentration tested, 128 µg/mL. The molecular weight of flambic acid is approximately 40% that of avilamycin (527 versus 1404). If the MIC concentrations are expressed as molar concentration, then the highest concentration of flambic acid tested (128 µg/mL or 0.24 mM) is approximately 40 times greater than the highest MIC for avilamycin (8 µg/mL or 0.006 mM). That is, the pharmacological activity of flambic acid is at least 40 times less than that of avilamycin.

Appendix K - Study S-AAC-82-04: Hydrolysis of avilamycin in buffer solution. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Sterile, aqueous buffer solutions of pH 5, 7, and 9 were fortified with 1.25 mg/L avilamycin and maintained in the dark at 24°C. Periodically samples were taken for analysis using an agar plate method and the organism *Micrococcus flavus* to measure bioactivity.

Results:
Avilamycin degraded in all solutions studied.

	pH 5	pH 7	pH 9
Half-life (hours)	12	230	52

Appendix L - Study S-AAC-82-04: An aqueous photolysis rate study with avilamycin. Study Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Sterile buffer solutions of avilamycin (1.25 mg/L) were prepared in pH 7 buffer and placed in 20-mL ampoules. The ampoules were irradiated using fluorescent sunlamps and black lights with an ultraviolet spectral energy distribution similar to natural sunlight. Samples were withdrawn for analysis after 1, 2, 4, 5, and 7 hours using an agar plate method and the organism *Micrococcus flavus*. Two ampoules were wrapped in aluminum foil and placed in the irradiation apparatus for seven hours as positive controls.

Results:
No significant degradation was observed in the control samples. In the irradiated samples, least squares analysis of the data obtained resulted in a first-order rate constant of -0.59 hr^{-1} and a half-life of 1.2 hours. Photolysis is likely to be a significant mode of dissipation in the environment.

Appendix M - Study EWD8429: Photodegradation of avilamycin in sunlight. Study Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Test Article: Avilamycin

Methods:
Solutions of avilamycin at 2 to 10 mg/L in 0.02 M pH 7 buffer, contained in quartz tubes, were exposed to summer sunlight. Avilamycin concentrations were determined at initiation and at 2, approximately 7 (0.5 days), and approximately 14 hours (1 day) using an agar plate method and the organism *Micrococcus flavus*. The sunlight intensity during the studies was monitored using the chemical actinometer p-nitroacetophenone.

Results:
Avilamycin degraded rapidly during the experimentation. In most samples exposed to sunlight for approximately 7 and 14 hours, no avilamycin activity was detected. Control samples of avilamycin incubated in the dark did not demonstrate degradation.

In one sample set, the concentration of avilamycin decreased from 10.0 mg/L at initiation to 0.258 mg/L after 7 hours (0.5 days). The first order degradation rate in this sample was 7.24 $days^{-1}$ corresponding to a half-life of 0.0958 days. Using the PNAP concentration to determine the sunlight intensity, avilamycin half-lives for other latitudes in summer and winter were calculated:

Latitude (Degree North)	Half-Life (Days)	
	Summer	Winter
20	0.086	0.150
30	0.091	0.236
40	0.096	0.475
50	0.110	1.33

Appendix N - Study ABC-0235: Field Dissipation of ^{14}C avilamycin swine manure metabolites. Study Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: Manure from pigs fed 90 ppm ^{14}C avilamycin

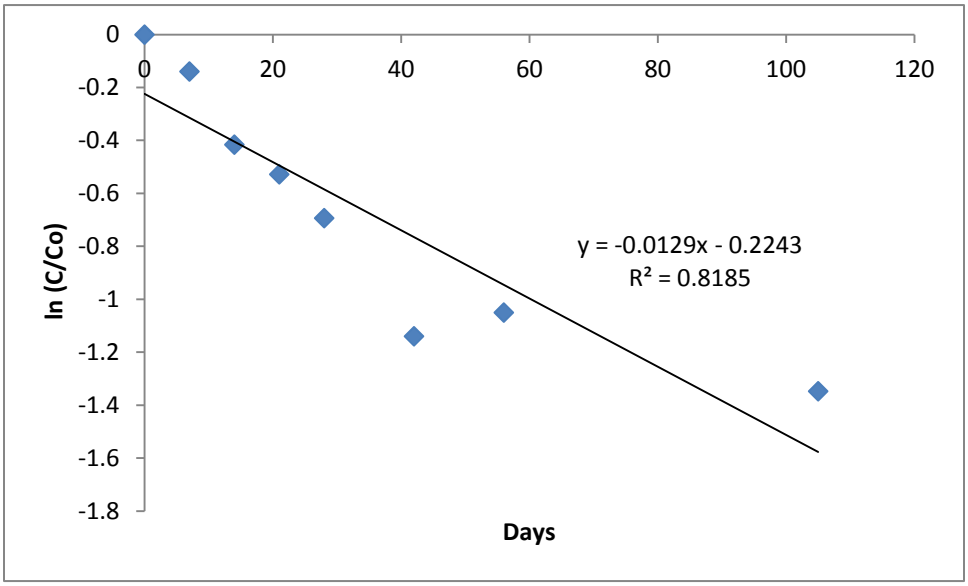
Methods:
Manure from swine fed 90 ppm of ^{14}C avilamycin was mixed with the top 5 cm of soil contained in a 0.65 m^2 galvanized steel ring buried in soil (silty loam, pH = 6.6) to approximately 0.75 m. At each sampling time, six core samples were taken at random and divided into 7.5 cm segments. Each corresponding 7.5 cm segment from the six cores was pooled and treated as a composite sample. Total radioactivity in the composite samples was measured by combustion.

Results:
The initial 0-7.5 cm core sample contained most of the radioactivity but some leaching did occur. Avilamycin-related substances in swine manure dissipated quickly and almost completely from soil without extensive leaching based on the low likelihood of leaching during this study due to very low precipitation during the first few weeks of this study (less than 1 cm prior to the Week 3 sampling interval). Within 4 weeks less than 50% of the initial radioactivity was accounted for in the soil.

Time (weeks)	% Initial Radioactivity			
	Core Segment (cm)			
	0-7.5	7.5-15	15-22.5	22.5-30
0	100	NT	NT	NT
1	79	8	NT	NT
2	53	13	NT	NT
3	51	8	13*	10*
4	43	4	2	<1
6	28	2	1	<1
8	30	3	2	NT
15	22	2	<1	<1
36	19	3	<1	<1
52	15	3	<1	<1

"NT" = not tested
*=samples appeared to be contaminated

As a post-study analysis of the data, a dissipation rate constant was calculated for data from the first 15 weeks of this study assuming first order degradation. This analysis omitted the samples that were considered to be contaminated. The rate was 0.013 $days^{-1}$ and the half-life was 53 days. A fairly uniform dissipation rate in the first 15 weeks was indicated by the significant correlation coefficient for the dissipation model (0.82). Uniform dissipation would not be expected if a substantial proportion of a compound leached out of the sampling zone during large random rainfall events.



Appendix O - Study 66679: Avilamycin: Aerobic transformation in four soils. Report Date: 2012.

Performing Laboratory: ABC Laboratories Inc.

Guidelines: GLP, OECD Guideline 307

Test Article: ^{14}C Avilamycin, the radiolabel is distributed uniformly throughout the molecule

Methods:
Four soils were fortified with 1 mg/kg ^{14}C-avilamycin and incubated under aerobic conditions at 20°C in the dark for up to 120 days with trapping of effluent gases in KOH. At sampling timepoints, evolved $^{14}CO_2$ was measured by liquid scintillation counting of the KOH traps, soils were extracted, and post-extracted soils were combusted. The radioactivity in the soil extracts was profiled by HPLC with radiometric detection. Major degradation products were isolated by fractionation and identified by LC/MS/MS.

The following summarizes the soil characteristics:

Name	USDA Textural Class	pH	% Organic Matter
Audrain	Loam	5.9	5.1
Tift	Loamy Sand	5.1	1.3
MSL-PF	Sandy Clay Loam	6.8	2.0
Raymondville	Sandy Clay Loam	8.0	0.83

Results:
Avilamycin showed rapid degradation in the four soils evaluated. After 7 days only 11% or less of the dosed avilamycin was detected in all four soils. A varying amount of avilamycin was mineralized to CO_2. At the end of incubation the amount of radioactivity in the KOH traps ranged from 15.1 to 58.0% of the applied radioactivity (AR).

There was a large amount of nonextractable residue observed in the study. Since the three soils with the highest amount of mineralization (27% to 58.0% AR) were also the soils with the largest amount of AR as nonextractable residue (31.7 to 37.1% AR), it is likely that the non-extractable residue was composed of small degradation products that had been incorporated in to the microbial-soil matrix.

Mass Balance and Transformation Kinetics:

	Audrain		Tift		MSL-PF		Raymondville	
Day	**7**	**120**	**7**	**120**	**7**	**120**	**7**	**51**
Mass Balance*	100.7	99.5	99.6	95.6	101.2	93.8	101.2	102.1
$^{14}CO_2$*	2.6	15.1	2.4	27.7	1.5	42.7	2.2	58.0
Nonextractable Residue*#	7.9	15.3	7.4	31.7	9.9	27.8	13.0	37.1
Extractable Radioactivity*	90.2	69.1	89.8	36.1	89.7	23.2	85.9	7.1
Avilamycin*$	1.8	0.3	10.0	0.4	7.5	0.1	10.9	1.1
DT50 (days)	0.2		1.5		0.4		1.0	
DT90 (days)	0.6		5.1		1.2		28.6	

*All values are percent of applied radioactivity
#By combustion
$In extract
DT50 and DT90 values are for avilamycin

Six major degradation products were observed over the course of the study. The degradation products suggest that there are two degradation pathways of avilamycin. In the primary pathway, hydrolysis leads to a cleavage of the avilamycin to the smaller flambic acid (M4 in the table below) which is can convert to flambalactone (M3) and the remainder of the avilamycin molecule (M2). In the other degradation pathway, which was observed in the Audrain soil more than in the other three soils, the eurekanate portion of avilamycin is removed to form M5.

Summary of the observed transformation products, with the maximum amount reached and the day of the maximum amount observed:

Product	Maximum % Applied Radioactivity observed in various soils			
	Audrain	**Tift**	**MSL-PF**	**Raymondville**
Avilamycin	75.7 (Day 0)	86.0 (Day 0)	87.4 (Day 0)	88.7 (Day 0)
M1	62.4 (Day 1)	19.2 (Day 10)	20.0 (Day 14)	26.4 (Day 3)
M2	51.1 (Day 2)	45.5 (Day 7)	58.2 (Day 3)	39.4 (Day 7)
M3	15.4 (Day 73)	15.7 (Day 10)	< 10	< 10
M4	13.8 (Day 2)	16.4 (Day 3)	23.7 (Day 3)	< 10
M5	22.9 (Day 51)	< 10	< 10	< 10
M6	27.9 (Day 120)	< 10	< 10	< 10

In general, the major degradation products of avilamycin were further degraded in the soil.

Appendix P - Study EWD8609: Soil adsorption of avilamycin. Report Date: 1986.

Performing Laboratory: Lilly Research Laboratories

Guidelines: Similar to OECD 106

Test Article: ^{14}C Avilamycin obtained by fermentation starting with ^{14}C-diethylmalonate as a precursor; more than 85% of the label is in the dichloroisoeverninic moiety

Methods:
A soil adsorption study was conducted with ^{14}C avilamycin in sandy loam, loam, and clay loam soils. Ten gram portions of the soils were equilibrated for 16 hours with 40 mL portions of 0.01 M $CaCl_2$ and ^{14}C avilamycin in 50 mL centrifuge tubes. After centrifuging, the avilamycin content of the aqueous layer was determined radiochemically. The amount adsorbed to the soil was determined by difference.

The pH value of 0.01 M $CaCl_2$ was brought o 7.3 by autoclaving for 20 minutes and then boiling for an additional 10 minutes to remove dissolved CO_2. Soilless controls were included to monitor adsorption to the glass vessels. Approximately 5% was found to bind to the vessels without soil. It was assumed that this amount also bound to the vessels when soil was added, thus, the amount assumed to adsorb to glass was subtracted from the amount bound to soil.

Results:

Soil	pH	% Organic Matter	% Organic Carbon[1]	Kd	Koc[2]
Sandy loam	4.9	2.6	1.5	51	3400
Loam	6.7	2.2	1.3	23	1769
Clay loam	7.0	5.1	3.0	109	3633

[1]Soil organic matter contains approximately 58% organic carbon, %OC was calculated by multiplying %OM by 0.58.
[2]Koc was calculated by dividing Kd by the fraction of organic carbon.

The Kds were based on total radioactivity. Given the pH values of the soil, it is likely that there was some degradation of avilamycin over the 16 hours of the equilibration study.

Appendix Q - Study 66678: Avilamycin: Determination of Adsorption/Desorption Using the Batch Equilibrium Method. Report Date: 2012

Performing Laboratory: ABC Laboratories Inc.

Guidelines: GLP, OECD Guideline 106

Test Article: ^{14}C Avilamycin, the radiolabel is distributed uniformly throughout the molecule

Methods:
Tier 1: Preliminary method development: The stability, sorption to filters and vessels, and solubility of avilamycin were investigated along with various soil:solution ratios. During the preliminary work, changes to the protocol were made to overcome the instability of avilamycin in acidic soils, the low solubility of avilamycin in acidic media, and the tendency of avilamycin to adsorb to filters and vessels. These changes were implemented in Tier 2 and included boiling and adjusting the 0.01 M $CaCl_2$ to pH 7 and preconditioning the vessels to reduce adsorption of avilamycin to the vessels.

Tier 2 Kinetic adsorption: ^{14}C-avilamycin was equilibrated with 5 soils suspended in 0.01 M $CaCl_2$ in pre-conditioned glass tubes at an initial aqueous concentration of 1 µg/mL. At 0.25, 1, 2, 4, and 20 hours, duplicate tubes were sacrificed and the phases separated by centrifugation. Partitioning between soil and water was calculated by measuring total radioactivity in the aqueous supernatant and calculating the amount in the soil by subtracting the supernatant radioactivity from what was originally added. Additionally, the soils were extracted and both the soil extract and the aqueous phase were analyzed via fractionation with radiometric detection. Based on the fractionation data, the partition coefficient specific for avilamycin was determined.

Tier 3: Isotherm test with the Raymondville soil (in which avilamycin was stable): Varying concentrations of ^{14}C-avilamycin (0.01 to 1 µg/mL in the aqueous phase) were equilibrated with Raymondville soil suspended in 0.01 M $CaCl_2$ (1:5 soil:solution ratio). Partitioning between phases was evaluated after a 2-hour adsorption step followed by a 2-hour desorption step.

Soils Used:

Name	USDA Textural Class	pH[a]	% Organic[b] Matter
Tift	Sand	5.2	1.1
Tehama	Loam	6.0	2.9
Audrain	Silty Clay Loam	6.3	5.4
Raymondville	Sandy Clay Loam	8.0	0.89
MSL-PF[c]	Sandy Clay Loam	6.8	2.0

[a] pH in 1:1 soil:water ratio

[b] Determined by Walkley-Black method

[c] Mutcher Sandy Loam-Pesticide Free

Results:

Avilamycin was found to degrade rapidly in all soils except the most alkaline sandy clay loam (Raymondville), thus, equilibrium could not be established. Partitioning coefficients were determined for total radioactivity and also for avilamycin. The Kd and Koc values presented below for each soil were determined using all timepoints for which a value was calculated.

Soil	pH	%OM	%OC	Total Radioactivity		Avilamycin	
				Kd	Koc	Kd	Koc
Sand	5.2	1.1		3.57	559	3.87	605
Loam	6.0	2.9		56.0	3321	22.3	1325
Silty clay loam	6.3	5.4		66.5	1987	16.9	537
Sandy clay loam	8.0	0.89		2.05	397	2.01	388
Sandy clay loam	6.8	2.0		143	11952	29.3	2444
Average over all five soils				**54**	**3643**	**15**	**1060**

Appendix R - Study ABC-0337: Evaluation of the soil mobility of avilamycin and its major fecal metabolite by soil thin-layer chromatography. Report Date: 1986.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: ^{14}C Avilamycin obtained by fermentation starting with ^{14}C-diethylmalonate as a precursor

Methods:
Soil TLC plates were prepared using coarse, medium and fine-textured soils. Radiolabeled herbicides trifluralin, atrazine, and dicamba were used as reference compounds representative of immobile, intermediately mobile, and highly mobile chemicals, respectively. ^{14}C Avilamycin and ^{14}C Metabolite A (flambalactone) were applied to to soil TLC plates along with the reference compounds. Plates were developed in water and radioautographs were prepared. Frontal R_f values of test and reference compounds were determined.

Results:
Avilamycin exhibited low mobility on all three soils. It was more mobile than triflualin but considerably less mobile that atrazine or dicamba. Using the soil mobility classification system described by Helling and Turner (1968), avilamycin would be considered a class 2 (low mobility) compound.

On the medium and fine-textured soils, flambalactone was much more mobile than avilamycin. Compared to the reference standards on these two soils, flambalactone was more mobile than atrazine but less mobile than dicamba. On the coarse soil, flambalactone exhibited low mobility of Metabolite A which could be due to the low pH of the soil (pH 4.9) and to the chemical nature of the metabolite itself (a lactone which can be readily hydrolyzed to the corresponding carboxylic acid). Using Helling and Turner's classification system, flambalactone would be considered a mobile compound (class 4 or class 5) on nonacidic soils and a compound of low mobility (class 2) on acidic soils (pH less than 5.0).

Helling CS, Turner BC. 1968. Pesticide Mobility: Determination by Soil Thin-Layer Chromatography. Science 162:562-563.

Soil thin-layer R_f values for test and reference compounds

	R_f Value				
Soil Type	Trifluralin	Avilamycin	Atrazine	Flambalactone	Dicamba
Coarse	0.07	0.09	0.48	0.27	0.88
Medium	0.05	0.26	0.58	0.84	0.98
Fine	0.07	0.12	0.68	0.95	1.00

Appendix S - Study S-AAC-82-04: A four-soil laboratory leaching study with avilamycin and an aged soil leaching study with avilamycin. Report Date: 1983

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{36}Cl Avilamycin

Methods:
Radiolabeled ^{36}Cl avilamycin was applied to the top of soil columns prepared with sand, sandy loam, loam, and clay loam soils. Each column was 30 cm by 1.0 cm i.d. The columns were leached with the equivalent with 60 cm water. Leachate was collected in 10-cm fractions and was analyzed radiochemically. At the end of the leaching process, the columns were broken into 5-cm sections, the sections were extracted, and the extracts and leachates were analyzed radiochemically.

In a second study to determine the mobility of soil degradation products, a sandy loam soil fortified with 7.6 µg/g ^{36}Cl avilamycin was aged 30 days. After 30 days, the soil was applied to the top of a 30 cm by 6.35 cm (i.d.) soil column. The column was leached with the equivalent of 1.25 cm rainfall per day for 45 days. At the end of the experiment, the column was broken into 5-cm sections, and the soil sections and leachate fractions were analyzed radiochemically.

Results:
Radioactivity leached in all four soils employed in the first part of the study. Total amounts of applied radioactivity found in the leachate were 22.1, 53.3, 26.7 and 22.7 % found for the sand, sandy loam, loam, and clay loam soils, respectively. Significant radioactivity was found in all soil sections of the columns. The results of the study indicate that avilamycin or its degradation products leached under the conditions employed and suggest that avilamycin or its hydrolysis products has potential for leaching in the environment.

The results of the part of the study that looked at radioactivity after incubation in soil, indicate that avilamycin and/or soil degradation products are susceptible to leaching. A total of 84.4 % of the radioactivity was found in the leachate. Only 11.8% of the applied radioactivity remained on the column and the majority of it was in the top section. A total of 96.2% of the applied radioactivity was accounted for at the end of the experiment.

Appendix T - Study 70541080: Effects of crystalline avilamycin on the activity of the soil microflora in the laboratory. Report Date: 2012.

Performing Laboratory: IBACON GmbH

Guidelines: GLP, OECD Guidelines 216 and 217

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Three concentrations of avilamycin in a loamy soil (1, 5, and 15 mg avilamycin/kg soil (dry weight)* were evaluated for effects on carbon and nitrogen transformation compared to untreated controls. Soil for the nitrogen transformation test was amended with lucerne meal prior to dosing with avilamycin. The concentration of avilamycin in the dosing solution (acetone) was confirmed by HPLC with uv detection. The soils were divided into three replicates per concentration for each test (carbon and nitrogen) and were incubated in plastic boxes at 20 to 23°C.

For carbon transformation, at 0, 7, 14, and 28 days after treatment with avilamycin, soil samples were removed in triplicate and supplemented with glucose. CO_2 production was measured for 24 hours following glucose addition.

For nitrogen transformation, at 0, 7, 14, 28, 42, and 56 days, the soil was sampled and analyzed for nitrate. The nitrogen transformation rate was calculated from nitrate levels at the time point compared to the nitrate levels at Day 0.

Results:
Carbon Transformation:

		Treatment Level		
	Control	1 mg/kg*	5 mg/kg	15 mg/kg
DAY 28 Respiration Rate mg CO_2/kg dry soil per hour	11.9	11.9	11.5	11.4
% Deviation from Control		-1	-4	-4

Nitrogen Transformation:

		Treatment Level		
	Control	1 mg/kg	5 mg/kg	15 mg/kg
DAY 28 Nitrate Content	32.9	33.4	36.4	38.6
DAY 28 Nitrogen Transformation Rate mg/kg dry soil per day	0.58	0.60	0.68	0.78
% Difference from Control		3	17	34
DAY 56 Nitrate Content	63.8	---	---	69.0
DAY 56 Nitrogen Transformation Rate mg/kg dry soil per day	0.84	---	---	0.93
% Difference from Control		---	---	11

The respiration rates in the soil treated with avilamycin up to 15 mg/kg were within 25% of the control soil.

Therefore, exposure to avilamycin has no impact on respiration activities of soil microorganisms up to 15 mg avilamycin/kg.

During the early weeks of the study, the soils treated with avilamycin had an increased amount of nitrate content compared to the control soil. The increase was concentration-dependent. On Day 28, the nitrogen transformation rate in the highest avilamycin treatment (15 mg/kg) was increased over the control by 34%. At 1 and 5 mg/kg, the nitrogen transformation rates were less than 25% different from the control. Therefore, the control and the highest avilamycin-treated soil were continued in incubation and evaluated at Day 42 and Day 56. By Day 56, the nitrogen transformation rate in the highest avilamycin treatment was increased over the control by only 11%.

Exposure to avilamycin results in transient increases in nitrogen transformation over the control. By Day 28, those increases are less than 25% that of the control in the 1 and 5 mg avilamycin/kg soil (dry weight). By Day 56, the increase in nitrogen transformation rate in the highest concentration is less than 25% that of the control. Therefore, avilamycin has no lasting impact on nitrogen transformation activities of soil microorganisms.

*Test concentrations given as mg avilamycin/kg soil (dry weight)

Appendix U - Study S-AAC-82-19: The effect of avilamycin on nitrification in soil. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Manure from swine fed a diet containing 80 mg/kg avilamycin

Methods:
Swine feces were collected from pigs on a basal ration with and without 80 mg/kg avilamycin as the dried fermentation product. A soil classified as a loamy sand was fortified with 400 mg/kg ammonium nitrogen by means of spraying with an aqueous solution of ammonium sulfate. Dried feces were mixed into the soil at two rates equivalent to 15 and 30 grams wet weight manure/kg dry soil. These rates are equivalent to manure application rates of 5 and 10 tons per acre when incorporated 2 inches deep which are equivalent to 13,658 and 27,315 kg wet weight manure/kg dry soil when incorporated 6 inches (or 15 cm) deep. Two controls were included, soil without feces and soil amended with 30 grams wet weight manure/kg soil from pigs not treated with avilamycin. The soil-manure mixtures were added to pots and watered to a moisture level of 75% of saturation.

The avilamycin activity in the treated manure was determined at initiation of the study using a microbiological agar plate method using *Micrococcus flavus* as the indicator organism. Based on microbiological activity in the feces, when the manure was applied to soil, the soil-manure mixtures contained 0.83 and 1.66 mg avilamycin per kilogram dry soil.

At initiation and at 1, 2, 3, 4, and 5 week intervals, soil samples were removed and analyzed for ammonium and nitrate to determine if nitrification, the process of ammonium oxidation to nitrate, was affected by avilamycin.

Results:
During the early weeks of this study, avilamycin demonstrated a concentration-response relationship with regard to the inhibition of nitrification in soil. The presence of manure alone in the soil also inhibited nitrification. The effect of avilamycin and manure appears to be transient. By 4 weeks, the amount of ammonia remaining in the samples is less than 10% of the initial values in all treatments. Also, at 4 weeks, all treatments have reached an approximate plateau in nitrate levels and neither avilamycin treatment is more than 25% different from either control.

Treatment		Ammonium Nitrogen (ppm)					
	Soil Rep	0 wk	1 wk	2 wk	3 wk	4 wk	5 wk
Manure-free soil	1	287	238	98	4	6	1
	2	280	255	121	10	7	1
30 grams/kg* control manure	1	364	236	170	34	18	4
	2	374	234	127	24	12	3
15 grams/kg* avilamycin manure	1	330	263	119	20	10	2
	2	328	272	138	12	12	3
30 grams/kg* avilamycin manure	1	338	316	230	46	12	3
	2	357	326	258	51	12	3

*kg of dry soil

Treatment		Nitrate Nitrogen (ppm)					
	Soil Rep	0 wk	1 wk	2 wk	3 wk	4 wk	5 wk
Manure-free soil	1	32	54	259	363	362	375
	2	32	65	244	384	397	394
30 grams/kg* control manure	1	18	10	101	263	315	310
	2	13	16	104	248	339	389
15 grams/kg* avilamycin manure	1	29	30	226	407	406	406
	2	22	42	210	389	331	388
30 grams/kg* avilamycin manure	1	13	11	68	316	365	395
	2	25	12	65	346	408	425

*kg of dry soil

The following table shows further post study analysis of the nitrate data included in the report to calculate the effects as percent differences from the controls.

Treatment	Average Nitrate Nitrogen at 4 weeks	% Difference from Manure-Free Soil	% Difference from Manure Control Soil
Manure-free soil	379.5	--	--
30 g/kg control manure	327	-13	--
15 g/kg avilamycin manure	368.5	-3	+13
30 g/kg avilamycin manure	386.5	2	+18

Appendix V - Study ABC-0263: Determination of the effect of avilamycin on seed germination. Report Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: Crystalline mixture of avilamycin factors (72.8% A, 9.9% B, 3.2% A', 2.1% C, 1.1% D1, 0.5% D2, 0.1% E and 10.3% unidentified components)

Methods:
Aliquots of 20 mL of distilled water containing avilamycin were added to two layers of filter paper in a petri dish. There were four replicates of each avilamycin treatment: 0.274 mg/petri dish, 0.548 mg/petri dish, and 1.096 mg/petri dish. There were also 4 replicates of a control treatment with just distilled water. Seeds of corn (*Zea mays*), wheat (*Triticum aestivum*), soybean (*Glycine max*), pinto bean (*Phaseolus vulgaris*), sweet pepper (*Capsicum annuum*), and tomato (*Lycopersicon esculentum*) were placed on the filter paper and germinated in the dark for 4 to 7 days at 25°C. Seeds were monitored for germination.

The treatment levels were chosen to expose the seeds to the amount of activity in manure from swine fed 40 ppm avilamycin and surface applied at rates of 20, 40, and 60 metric tons of manure/hectare. These rates are equivalent to 8094, 16188, and 24282 kg manure/acre for 40 ppm avilamycin, or 4047, 8094, and 12141 kg manure/acre for pigs fed 80 ppm avilamycin.

Results:
Seeds of all six plant species treated with avilamycin at all treatment levels had germination rates similar to that of the control seeds.

	% Germination					
mg/dish	Pinto Bean	Soybean	Corn	Wheat	Sweet Pepper	Tomato
0	62.0	72.0	90.0	68.3	66.7	91.7
0.274	68.0	72.0	94.7	72.7	64.0	82.7
0.548	75.0	78.0	92.8	65.0	65.0	88.3
1.096	62.0	75.0	85.3	79.3	62.7	87.3

Appendix W - Study ABC-0261: Phytotoxicity study with manure from swine treated with avilamycin. Report Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: Manure from swine fed a diet containing 300 mg/kg avilamycin

Methods:
Corn (*Zea mays*), tomato (*Lycopersicon esculentum*), soybean (*Glycine max*), and wheat (*Triticum aestivum*) plants were grown from seed in pots containing soil dosed with dried manure from swine maintained on a control ration or a ration containing avilamycin. Each experimental pot was dosed with 3 g of dried manure. The pots were six inches in diameter (28.3 in^2 surface area) containing soil 6 inches deep. The manuring rate was 1642 kg/acre:

3 g dry feces ÷ 40.6% dry matter original sample = 7.39 g wet
28.3 in^2 = 0.0000045 acre per pot (1 in^2=1.59 x 10^{-7} acre)
0.00739 kg ÷ 0.0000045 acre = 1642 kg/acre

Since the pigs in this study were fed 300 ppm avilamycin in the feed, which is 3.75 times greater than the 80 ppm that is being considered in the current assessment, the manuring rate is equivalent to 6158 kg/acre of manure from pigs fed 80 ppm avilamycin.

Measurements of shoot height were made 14 and 21 days after treatment. At the termination of the test, 21 days after seeding, the shoots were cut at the soil line and the fresh weight of both the shoots and the roots (as composite samples) were measured.

Results:

	Height of Plants (cm)			
	14 Days		21 Days	
Plant Type	Control	Avilamycin	Control	Avilamycin
Corn	38.6	39.9	60.6	61.9
Wheat	24.4	23.7	30.7	30.0
Soybean	11.0	10.8	16.8	16.1
Tomato	3.0	2.9	7.8	7.8

	Mean Weight/Plant (g)			
	Shoots		Roots	
Plant Type	Control	Avilamycin	Control	Avilamycin
Corn	7.61	7.99	5.55	4.05*
Wheat	0.95	0.92	0.44	0.43
Soybean	3.16	3.00	2.52	2.62
Tomato	1.41	1.48	0.10	0.19*

*Significantly different from control using t-test

Manure from avilamycin-treated swine had no effect on the shoot height or weight in any of the four plant species evaluated. In the treatment replicates, the root weight for corn was decreased by 27% compared to control and this difference was significant (t-test, $p<0.05$). On the other hand, the root weight for tomatoes was almost doubled in the treatment group compared to the control which was also significant (t-test, $p<0.05$). Given the difficulty in removing the root mass completely and cleanly from soil, it is unclear if the decrease in root weight in corn is meaningful. Additionally, data from a more recent study which tested much higher concentrations in the soil, found no emergence or growth effect on corn.

Appendix X - Study 66369: Effects of avilamycin on the seedling emergence and early seedling growth of terrestrial plants following OECD guideline 208. Report Date: 2012.

Performing Laboratory: ABC Laboratories Inc.

Guidelines: OECD 208, GLP

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
A 21-day seed emergence and growth study was conducted to determine the effects of avilamycin on corn (*Zea mays*), soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), oat (*Avena sativa*), radish (*Raphanus sativus*), and sugar beet (*Beta vulgaris*). A sandy loam soil was fortified with avilamycin at concentrations of 7.79 (tomato only), 15.6, 31.2, 62.5, 125, 250, and 500 mg avilamycin activity/kg soil (dry weight). Soil fortification was achieved by dissolving avilamycin in acetone, applying the acetone dosing solution to sand, and then mixing the sand with the soil. The concentrations of avilamycin in the acetone dosing solutions were confirmed analytically by HPLC/uv. A blank control and a solvent control were included in the study. There were six replicate pots per treatment level with five seeds planted in each pot. The study was conducted in a greenhouse and over the course of the study, the average temperature was 28.4°C, the average relative humidity was 81%, and the average daily light was 497 $\mu E\ m^{-2}s^{-1}$. Emergence, survival, visual injury, shoot length and replicate shoot weight were evaluated as endpoints.

Results:
There were no significant trends in emergence, survival, shoot length or shoot weight following treatment with avilamycin. Therefore, LC50 for survival was >500 mg/kg soil (dry weight) for all species. The EC50 values for emergence, shoot length, and shoot weight were >500 mg/kg soil (dry weight) for all species tested. The no-observed-effect concentrations for all endpoints in all species were equal to 500 mg/kg soil (dry weight).

Emergence and Survival				Avilamycin mg/kg in soil (dry weight)						
	Blank	**Vehicle**	**Pooled**	**7.79**	**15.6**	**31.2**	**62.5**	**125**	**250**	**500**
Corn										
% Emergence	97	97	97	--	100	100	100	97	100	97
% Survival	100	100	100	--	100	100	100	100	100	100
Oat										
% Emergence	87	100	93	--	100	97	87	100	93	100
% Survival	100	100	100	--	100	100	100	100	100	100
Radish										
% Emergence	100	97	98	--	87	97	97	97	93	97
% Survival	100	97	98	--	100	100	100	100	100	100
Soybean										
% Emergence	100	90	95	--	100	97	90	93	97	93
% Survival	100	96	98	--	100	100	100	96	100	100
Sugar Beet										
% Emergence	90	97	93	--	93	97	93	100	90	100
% Survival	100	100	100	--	100	100	100	90*	96	100
Tomato										
% Emergence	90	97	93	90	84*	97	97	97	86	97
% Survival	100	97	98	100	100	100	97	100	100	100

*These were significantly lower than the pooled control. However, given the lack of dose response these decreases were not considered to be related to avilamycin.

Individual Shoot Length and Replicate Shoot Weight				Avilamycin mg/kg in soil (dry weight)						
	Blank	**Vehicle**	**Pooled**	**7.79**	**15.6**	**31.2**	**62.5**	**125**	**250**	**500**
Corn										
Shoot length mm	752 (43.9)	739 (68.1)	746 (21.8)	--	785 (57.1)	745 (44.1)	750 (53.0)	742 (48.0)	765 (41.9)	734 (60.5)
Shoot weight g	3.445 (0.560)	3.517 (0.441)	3.481 (0.441)	--	4.116 (0.288)	3.320 (0.334)	3.628 (0.331)	3.610 (0.300)	3.461 (0.339)	3.141 (0.427)
Oat										
Shoot length mm	310 (20.4)	329 (12.1)	320 (8.28)	--	327 (37.7)	319 (25.0)	345 (20.0)	316 (15.3)	306 (21.6)	310 (12.4)
Shoot weight g	0.2439 (0.0626)	0.3304 (0.0645)	0.2872 (0.0577)	--	0.2752 (0.0240)	0.2901 (0.0281)	0.2704 (0.0766)	0.2871 (0.0391)	0.2580 (0.0467)	0.2711 (0.0374)
Radish										
Shoot length mm	134 (26.7)	149 (14.9)	142 (17.4)	--	150 (9.25)	142 (11.4)	142 (15.3)	132 (24.6)	142 (18.4)	143 (10.1)
Shoot weight g	0.4032 (0.122)	0.5256 (0.144)	0.4644 (0.0723)	--	0.4956 (0.0954)	0.5052 (0.0969)	0.5460 (0.110)	0.3907 (0.0671)	0.4631 (0.0769)	0.4328 (0.0698)
Soybean										
Shoot length mm	489 (59.9)	495 (97.7)	493 (70.0)	--	510 (58.5)	488 (58.0)	484 (112)	488 (103)	515 (50.2)	507 (64.6)
Shoot weight g	2.975 (0.202)	2.616 (0.417)	2.795 (0.185)	--	2.906 (0.275)	2.703 (0.342)	2.562 (0.283)	2.586 (0.397)	2.768 (0.157)	2.496 (0.323)
Sugar Beet										
Shoot length mm	114 (22.3)	132 (12.1)	123 (11.3)	--	129 (19.7)	138 (14.5)	150 (15.3)	109 (23.6)	124 (16.8)	130 (9.93)
Shoot weight g	0.2629 (0.105)	0.3318 (0.0440)	0.2973 (0.0644)	--	0.3028 (0.0454)	0.3337 (0.0612)	0.3863 (0.0643)	0.2194 (0.106)	0.2635 (0.0768)	0.3158 (0.0468)
Tomato										
Shoot length mm	94.6 (11.9)	93.3 (9.11)	93.9 (8.03)	86.6 (6.84)	88.1 (7.17)	90.9 (5.95)	88.8 (9.25)	95.5 (6.76)	88.2 (7.17)	85.3 (7.13)
Shoot weight g	0.1523 (0.0278)	0.1528 (0.0236)	0.1525 (0.0232)	0.1377 (0.0403)	0.1666 (0.0517)	0.1497 (0.237)	0.1544 (0.0240)	0.1727 (0.0165)	0.1303 (0.0294)	0.1403 (0.0168)

There were no significant differences in treatment groups compared to the pooled control.

Appendix Y - Study W01882: The toxicity of soil-incorporated avilamycin (EL-750, Compound 48740) to earthworms (*Lumbricus terrestris*) in a 14-day test. Revised Report Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Guidelines: GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
Test media (rabbit feces, loamy sand soil, and water) was placed in 2-L glass jars. Three replicate jars were used for each of the following: control media; media containing 10 mg/kg soil (dry weight)* of nominal avilamycin activity; and media containing 100 mg/kg of nominal avilamycin activity. These media levels are equivalent to 0.0, 67.1, and 671 mg of dried fermentation product/kg. Five worms, about 4 g each, were placed into each of the nine jars at the beginning of the study. The test was conducted at 12°C. On day 7 and day 14 of the study, the worms were weighed, examined and described as follows: normal, flaccid, soft and flaccid, moribund, or dead.

Results:
All worms were found to be normal throughout the study. No flaccid, soft and flaccid, moribund or dead earthworms were found in any control or treatment group. The average body weights of worms exposed to 10 and 100 mg/kg were 4.530 and 4.397 g, respectively, and these were not significantly different from the average weight of control worms, 4.448 g. The average body weight increased during the study. Percent average body weight increases for the worms were: control, 11.5%; 10 mg/kg treatment, 11.4%; 100 mg/kg treatment, 10.7%. The average weight gains of worms in the two treatment levels were not statistically different from the average weight gain of control worms.

The 14-day no-observed-effect concentration (NOEC) for avilamycin in earthworms was 100 mg/kg and the EC50 was >100 mg/kg.

*Test concentrations are given as mg avilamycin activity/kg soil (dry weight)

Appendix Z - Study 66370: Avilamycin: Survival and reproduction test with the earthworm, *Eisenia fetida*. Report Date: 2011.

Performing Laboratory: ABC Laboratories Inc.

Guidelines: OECD 222, GLP

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Artificial soil was fortified with nominal concentrations of avilamycin of 87, 170, 330, 670, and 1300 mg/kg soil (dry weight)*. Fortification was conducted using acetone to deliver avilamycin to sand and then mixing the sand into the artificial soil. A blank control and a vehicle control were included. The concentration of avilamycin in the acetone dosing stock solutions was confirmed using an HPLC/uv method. Adult worms (10 per replicate, 8 replicates each for the blank and solvent controls and 4 replicates per avilamycin treatment level) were incubated for 4 weeks under fed conditions. After 4 weeks adults were removed from soil, assessed for health and weighed. Vessels were incubated for an additional 4 weeks. After 4 weeks, reproduction was assessed by carefully sifting through the soil in each vessel and removing and counting offspring.

Results:
After 28 days of exposure, the percent mortality of adult worms was 3, 3, 0, 3, 0, 0, and 0% in the control, vehicle control, 87, 170, 330, 670, and 1300 mg/kg treatments. All of the live earthworms were observed to be normal.

The control worms lost an average of 17% in replicate mass during the 28-day exposure which the vehicle control worms lost an average of 13%. The percent change in replicate mass of worms in the avilamycin treatments ranged from a loss of 3% to a gain of 8%.

The average reproduction (% coefficient of variation) for the blank and vehicle control groups were 65 (27%) and 81 (27%). The mean number of juveniles per replicate in the pooled control (blank + vehicle) was 73 (% CV of 29%). The average number of juveniles per replicates was 78, 73, 66, 58, and 60 in the 87, 170, 330, 670, and 1300 mg/kg treatment levels which were 107%, 100%, 90%, 79%, and 82% of the pooled control value. While there was a trend towards reduced offspring with increasing concentration of avilamycin in the soil, there was no statistically significant reduction in the reproductive output of worms exposed to the test substance as compared to the pooled control reproduction.

The LC50 and EC50 values were estimated to be > 1300 mg avilamycin/kg. The highest concentration in which there was no statistically significant effect on earthworm survival, growth, and reproduction was 1300 mg/kg. Given the trend to decreased reproduction and the fact that the reproduction the highest two concentrations was decreased by more than 15%, the conservative NOEC will be considered to be 330 mg avilamycin/kg soil (dry weight).

*Test concentrations given as mg avilamycin activity/kg soil (dry weight)

Appendix AA - Study S-AAC-82-08: Avilamycin: Interaction with Sewage Microorganisms Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Avilamycin dried fermentation product, containing 16.7% avilamycin activity

Methods:
Inoculum from a sewage plant's aerated lagoon was treated with avilamycin as the dried fermentation product. Treatments were made daily by adding increasing concentrations of avilamycin up to a maximum of 102.6 mg/L. Total volume in the aeration vessels was held constant by removing a portion of the supernate before replacing it with nutrient solution. Aeration was accomplished with laboratory air flowing at 3 ft^3/hr.

The effect of avilamycin on sewage microorganisms was determined by measuring biochemical oxygen demand (BOD, 5 day), viable cell counts, pH and dry weights.

The concentration of avilamycin activity in the test system was measured using a microbiological assay.

Results:
Analyses made on treated systems were compared to non-treated controls.

After daily treatments, initial BODs were naturally high in all systems, but subsequent analyses showed the expected reduction of nutrients. Even at high avilamycin concentrations, the microbiological digestive activity was not inhibited.

Colony-forming units were lower in the avilamycin-treated samples in the first eleven days. These findings indicate that avilamycin may adversely affect the microbial population in the sewage system for the first two weeks, but it is clear that the effect is transient. pH and solids in treated samples were not different from untreated controls.

Confirmatory dissolved oxygen utilization tests with acclimated inocula indicated no inhibition of respiration of sewage microorganisms.

The highest concentration of avilamycin activity measured in the test systems was 87.6 mg/L.

Appendix BB - Study T4EFR0701: Blue-green algae (cyanobacterial) growth inhibition test. Report Date: 2007.

Performing Laboratory: CIT

Guidelines: GLP, OECD Guideline 201

Test Article: Crystalline avilamycin

Methods:
A static toxicity test was conducted to evaluate the effects of avilamycin on the cyanobacterial strain, *Synechococcus leopoliensis*, over a 72 hour exposure. Cell suspensions were exposed to nominal concentrations of 0.625, 1.25, 2.5, 5 and 10 mg avilamycin activity/L. Three replicates were included for each test concentration and six replicates for a dilution water control. Cell growth data was recorded at 24, 48, and 72 hours by means of a Malassez cell counter. Data was expressed as yield and average specific growth rate.

Results:
During the test, the pH ranged from 8.07 to 8.25 and the temperature ranged from 22.6 to 23.7°C. The test was conducted under continuous lighting at 3700 to 3880 lux. There was some evidence of precipitation in 2.5, 5, and 10 mg/L test solutions.

Exposure to avilamycin caused a decrease in growth rate at higher concentrations. The magnitude of the decrease was greatest at 24 hours when the percent inhibition compared to control was 16% and 41% in the 5 and 10 mg/L treatment levels, respectively. After 72 hours, the inhibition was 0, 0, 2, 7, and 20% compared to control in the 0.625, 1.25, 2.5, 5 and 10 mg/L, respectively. The growth rates at 5 and 10 mg/L was statistically significantly different from that of the control. The EC50 for growth rate was >10 mg/L and the no observed effect level was 2.5 mg/L.

Yield at 72 hours was inhibited by 0, 0, 11, 33, and 69% compared to the control at 0.625, 1.25, 2.5, 5, and 10 mg/L, respectively. The yields at 5 and 10 mg/L were statistically different from that of the control, but since the inhibition at 2.5 was 11%, the NOEC was conservatively considered to be 1.25 mg/L. The EC50 for yield was determined to be 6.85 mg/L.

Appendix CC - Study C03382: The acute toxicity of avilamycin (EL-750, Compound 48740) to *Daphnia magna* in a static test system. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Guidelines: EPA, ASTM, GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
A group of 30 and a group of 29 *Daphnia*, ≤24 hours old, were exposed to control water and to a nominal avilamycin activity concentration of 100 mg/L, respectively, for 48 hours. Each of the three replicate beakers contained 200 ml of test solution and were used to contain 9 or 10 daphnia for the treatment and control. Water samples were taken at 0 and 48 hours for analysis of the avilamycin concentration using an agar well method with *Micrococcus flavus*. Temperature, dissolved oxygen, and pH of the test solutions were measured at least at the beginning and end of the study. Total alkalinity, total hardness, and conductivity were measured for the dilution water. *Daphnia* were assessed for hypoactivity, prostration, and immobility.

Results:
The mean measured concentration of avilamycin in the test solution was 23.8 mg/L. No avilamycin was detected in the control solutions.

The water quality characteristics were as follows: pH, 7.3 to 8.0; dissolved oxygen concentration, at least 83% of saturation; temperature, 20.0˚C; total alkalinity, 148 mg/L (as $CaCO_3$); total hardness, 137 mg/L (as $CaCO_3$); and conductivity, 250 µmhos/cm.

No mortalities were found. No daphnia were hypoactive, prostrate or immobile in this study. The 48-hour no-observed-effect concentration (NOEC) for avilamycin in daphnia was 23.8 mg/L and the EC50 was >23.8 mg/L.

Appendix DD - Study 66272: Avilamycin: Acute toxicity to the water flea, Daphnia magna, determined under static test conditions following OECD guideline 202. Report Date: 2011.

Performing Laboratory: ABC Laboratories Inc.

Guidelines: OECD 202, GLP

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Daphnids, <24 hours old, were exposed to each nominal treatment level of control, 9.98, 20.1, 40.1, 80.3, and 160 mg avilamycin activity/L (nominal) for 48 hours. Each of the four replicate beakers at each treatment level contained 200 ml of test solution and was used to contain 5 daphnids. Water samples were taken at 0 and 48 hours for analysis of the avilamycin concentration using an HPLC/uv method. Temperature, dissolved oxygen, and pH were measured at test initiation and termination. Total alkalinity, total hardness, and conductivity were measured in the dilution water at test initiation. *Daphnia* were assessed for immobility and sublethal effects.

Results:
Given evidence of insolubility (cloudiness) at the two highest nominal concentrations, the analytical samples were centrifuged prior to analysis. The mean measured concentrations of avilamycin in the test solutions were 0, 9.67, 19.8, 39.1, 68.8, and 138 mg/L.

The water quality characteristics were as follows: pH, 8.2 to 8.6; dissolved oxygen concentration, at least 93% of saturation; temperature, 20.2 to 21.0˚C; total alkalinity, 156 mg/L (as $CaCO_3$); total hardness, 148 mg/L (as $CaCO_3$); and conductivity, 337 µS.

No mortalities or sublethal effects were observed. The 48-hour no-observed-effect concentration (NOEC) for avilamycin in daphnia was 138 mg/L and the EC50 was >138 mg/L.

In the analysis, avilamycin had a short retention time under the chromatography conditions used. Therefore, it is possible that some degradation products may have been present but were integrated under the peak of avilamycin. For C03382 the pH ranged from 7.3 to 8.0, while in Study 66272 the pH ranged from 8.2 to 8.6. Avilamycin is more stable at pH 7 than at pH 9, so there was more hydrolytic potential in Study 66272. Therefore, it is possible that the invertebrates could have been exposed to some concentrations of the degradation products of avilamycin.

Appendix EE - Study F12782: The acute toxicity of avilamycin (EL-750, Compound48740) to bluegill (*Lepomis macrochirus*) in a static test system. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Guidelines: ASTM, EPA, GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
Three groups of 10 juvenile bluegill (mean individual weight, 1.0 g) were exposed for 96 hours to nominal avilamycin concentrations of 0.0 and 100 mg/L (671 mg/L of dried fermentation product). Samples of test solutions were taken at 0, 24, 48, 72, and 96 hours for analysis for avilamycin concentration using an agar well method with *Micrococcus flavus*. Dissolved oxygen concentration, pH and temperature were recorded daily for each jar. Total hardness, total alkalinity, and conductivity were recorded once for the dilution water. Behavioral signs of toxicity and mortalities were noted for fish in each jar on a daily basis.

Results:
Analyzed concentrations of avilamycin throughout the test ranged from 30.1 to 41.8 mg/L for the three replicate 15 L treatment jars and the overall avilamycin level was 35.4 mg/L. Avilamycin was not detected in any of the three 15L control jars. Water quality characteristics were as follows: pH, 8.0 to 8.7; dissolved oxygen at least 93% saturation; temperature, 20.0˚C; total hardness, 120 mg/L (as $CaCO_3$); alkalinity, 140 mg/L (as $CaCO_3$); and conductivity, 225 µmhos/cm.

No mortalities occurred in this study. No behavioral signs of toxicity were noted for any fish in this study. The 96-hour no-observed-effect concentration (NOEC) for avilamycin in bluegill was 35.4 mg/L and the LC50 was >35.4 mg/L.

Appendix FF - Study F12682: The acute toxicity of avilamycin (EL-750, Compound 48740) to rainbow trout in a static test system. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Guidelines: ASTM, EPA, GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
Groups of 10 juvenile rainbow trout (mean individual weight, 1.09 g) were exposed to nominal concentrations of 0.0 (water control), 25, 50, 75, and 100 mg avilamycin activity/L for 96 hours. Jars with 15 L of test or control solution were used to contain each group of ten fish. Samples of test solutions were taken at 0, 24, 48, 72, and 96 hours for analysis of avilamycin concentration using an agar well method with *Micrococcus flavus*. Dissolved oxygen concentrations, pH, and temperature of the solutions were recorded daily. Total alkalinity, total hardness, and conductivity of the dilution water were determined. Behavioral signs of toxicity and mortalities were monitored for fish in each jar on a daily basis.

Results:
The mean measured concentrations of avilamycin for the five treatment levels were 0, 20.5, 39.8, 44.7, and 47.8 mg/L. Water quality characteristics were as follows: pH, 7.9 to 8.4; dissolved oxygen, at least 96% saturation in all test solutions; temperature, 12.0˚C; total hardness, 120 mg/L (as $CaCO_3$); total alkalinity, 140 mg/L (as $CaCO_3$); and conductivity, 225 µmhos/cm.

No mortalities occurred in this study. No behavioral signs of toxicity were noted for any fish in this study. The 96-hour no-observed-effect concentration (NOEC) for avilamycin in rainbow trout was 47.8 mg/L and the LC50 was >47.8 mg/L.